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Acute Toxicity and Mutagenicity of Peruvian Crude Oil and Oil-Contaminated Samples from the Peruvian Amazon

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Final Report

by

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ABSTRACT

ACUTE TOXICITY AND MUTAGENICITY OF PERUVIAN CRUDE OIL AND OIL-CONTAMINATED SAMPLES FROM THE PERUVIAN AMAZON

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Oil industry activities such as exploration, transportation, use and disposal are a major source of contamination in Peru and have significant deleterious effects on aquatic organisms and the environment. The objective of this study was to report LC_{50} values from acute toxicity tests on a native Peruvian fish species - red pacu, *Piaractus brachypomus* and fathead minnow, *Pimephales promelas*. This study also reports PAH concentrations, mutagenicity, and Microtox EC_{50} values of Peruvian crude oil, and water and sediment from the vicinity of two towns on the Marañón River and the Corrientes River in Loreto, Peru. Toxicity results showed that LC_{50} values on *Piaractus brachypomus* for three reference toxicants were: zinc sulfate = 5.74 mg/l, sodium dodecyl sulfate = 11.29 mg/l, and Louisiana sweet crude oil = 2.05 mg TPH/l. The LC_{50} for Peruvian crude oil was > 4.00 mg TPH/l, and the LL_{50} was found to be > 50,000 mg/l. The LC_{50} of Peruvian crude oil on *Pimephales promelas* was 1.83 mg TPH/l, while the LL_{50} was found to be 22,920 mg/l. The highest total PAH concentration was found in water from the Marañón River, 210.15 µg/ml. All water samples tested, and one sediment sample were found to be mutagenic ($P < 0.001$). The EC_{50} of a sediment sample from Marañón River was 335.1 mg/l, and at Corrientes River toxicity ranged from 25.67 to 133.86 mg/l. Peruvian crude oil was mutagenic using strain TA98 and S9 enzyme and the EC_{50} was 17.18 mg/L. The two areas sampled had very high PAH concentrations that are most likely associated with oil activities.

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CHAPTER 1 – INTRODUCTION

Oil industry activities such as exploration, transportation, storage, use and disposal are sources of major contamination problems. For instance in 2003, one of the world's largest integrated energy companies (Texaco), later bought by Chevron, was sued by Ecuadorian residents for dumping and spilling toxic waste and oil, abandoning waste pits, and burning gases in the Ecuadorian Amazonian rainforest in the 1970s. This case was settled, pending appeal (2010), when Chevron was ordered to pay an $8.6 billion fine (WSJ, 2011).

Crude oil has a complex chemical nature, inclusive of hundreds of different organic constituents. Most of them are hydrocarbons that consist of three major types: alkanes, cycloalkanes, and aromatics (Mason, 2002). Alkanes are a class of aliphatic hydrocarbons characterized by open chains of carbon atoms with only single bonds between adjacent carbon atoms. Simple alkanes include methane, ethane, propane, and hexane. Cyclohexanes are ringed alkanes with the molecular formula $C_nH_{2n.}$ They are rather unreactive, non-polar, not readily biodegradable and moderately toxic to aquatic organisms (Irwin, 1997). Aromatic hydrocarbons are composed of hydrogen and carbon, arranged in benzene rings, with low water solubility, and high lipophilicity (Maliszewska-Kordybach, 1999).

Pyrogenic and petrogenic are two types of anthropogenic PAH sources. Pyrogenic sources are formed by incomplete burning of fossil fuels such as coal, diesel, wood, and tobacco. Petrogenic sources include petroleum products, drilling operation effluents and crude oil transport and releases (Saha et al., 2009). Agricultural fires, domestic, and industrial wastes also release PAHs. Natural sources include sediment

erosion, oil seeps, forest fires and volcanic activity. None of these natural sources contribute significantly to the overall emission of PAHs (Mohammadi Zadeh et al., 2010; Maliszewska-Kordybach, 1999). However, the main environmentally hazardous sources are petroleum production, transportation activities, and drilling operations.

Aromatic hydrocarbons can be transported long distances in the water column because of their resistance to degradation, especially those with the highest molecular weight. This release not only causes acute mortality to organisms directly exposed to crude oil, but also to those organisms that may be near refining and transportation activities, as well as those further downstream (Simcik et al., 1996; Anyakora et al., 2008; Pérez et al., 2008). Because PAHs are stable and non-polar, upon later release by biological and physical disturbances, they accumulate more in organisms such as aquatic plants, fish, and invertebrates than in water or sediment (Anyakora and Coker, 2007).

Polycyclic aromatic hydrocarbons are absorbed by organisms during exposure to contaminated food, water, and sediments. Organisms that are exposed to oil pollution for a long period of time may be affected negatively; altering their growth, metabolism, and potential productivity. (Lapviboonsuk and Loganathan, 2007). For example, after the *Prestige* oil spill off the coast of Galicia, Spain in 2002, thousands of birds died. Studies were made on bird blood and it was determined that yellow-legged gulls *Larus michahellis* were altered physiologically including toxic and inflammatory effects and immune-suppression (Alonso-Alvarez et al., 2007). An example of carcinogen induction from PAH contaminated areas is the elevated incidence of liver tumors found in brown bullheads *Ameiurus nebulosus* (Pinkney et al., 2004) and bottom-dwelling marine flatfish (Dunn, 1991).

Short-term symptoms of PAH exposure in humans include nausea, diarrhea, eye irritation and vomiting. Skin contact to naphthalene can cause skin redness and inflammation (Ohio EPA, 2002). Chronic toxicity testing of PAHs in fish, mammals, etc. have found the following hydrocarbons to be carcinogenic or possible human carcinogens: benzo[a]anthracene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, dibenzo[a,h]anthracene, and indeno[1,2,3-cd]pyrene (IARC, 1983). Lung, liver, skin, stomach and bladder cancers on test animals have been reported (ATSDR, 2008). Given the propensity of PAHs to bioaccumulate in tissues, fish and other edible aquatic organisms that are exposed to PAH contamination endanger the public through consumption and represent an appreciable human exposure to carcinogens (Tuvikene, 1995). Therefore, it is necessary to test the toxicity of these contaminants and use the information as part of the basis for public health and regulatory decisions concerning toxic chemicals.

The test exposure of an organism to a stimulus is known as a bioassay and includes both bioaccumulation tests that measure the body burden of a pollutant, and toxicity tests which measure an effect of a pollutant (Chapman, 1995). The purpose of acute toxicity tests with fish is to compare them with other species' acute testing and to help to determine water quality criteria (USEPA, 1996a). The most sensitive fish life stages to xenobiotics are the early ones due to enzyme inhibition and tissue injury. Larval stages are more affected because they have a larger surface to volume area to uptake contaminants and their organs are not completely developed (Rodrigues et al., 2010). Fish embryos have shown abnormalities (deformities, erosions, lesions and tumors (DELTs)) in response to PAH exposure, including spinal curvature, edema and

reduction of craniofacial structures. Embryonic toxicity of PAHs is characterized by curvature of the body axis and jaw reduction. In addition, cardiac function defects have secondary negative effects such as cardiac morphogenesis, neural tube structure, and kidney development (Incardona et al., 2004; Nokame et al., 2008).

Acute toxicity bioassays are a prescreening tool for the chemical assessment of polluted water (De Zwart and Slooff, 1983). One such assay, the Microtox® toxicity assay is based on the inhibition of light emitted by the bioluminescent marine bacteria *Vibrio fischeri*, formerly known as *Photobacterium phosphoreum*. The Microtox® toxicity assay has been widely used due to its toxicity screening ability, reproducibility, and easy application (Beg and Ali, 2008). It has been used to detect the relative toxicity of fungi, such as *Aspergillus fumigatus* (Alba et al., 2009), pesticides (Ruiz et al., 1997), industrial waste (Hao et al., 1996), water-soluble crude oil fractions (Ziolli and Jardim, 2002), and oil contaminated soil and sediment (Loureiro et al., 2005, Blaise et al., 2004). Toxicity is expressed in terms of EC_{50}, a standard measurement of toxic effects that is defined as the effective concentration of a toxicant that would show a response in half of the individuals in an experiment (Berglind et al., 2010).

Wastewater containing oil contains harmful substances, including those with genotoxic effects that are described as any process that affects DNA structure (Bohne and Cathomen, 2008). Genotoxicity studies in Ecuador, on the Amazonian population close to crude oil extraction zones have shown DNA damage such as type B nuclei fragmentation and chromosomal aberrations (Paz-y-Miño et al., 2012). A distillate from Venezuelan crude oil was found to increase DNA adduct formation in rat liver (Nagy et al., 2004). Numerous spills and leakages involving petroleum have occurred in Brazilian

rivers and genotoxicity assays have also been performed. For instance, chromosomal aberration assays on *Allium cepa* exposed to petroleum polluted water showed breaks in chromosomes and changes in chromosome number (Leme et al., 2008). While, nuclear degeneration and bi-nucleated hepatocytes have been found in marine pejerrey *Odontesthes argentinensis* exposed to the water soluble fraction of diesel and gasoline (Rodrigues et al., 2010).

Mutagenicity is a critical step in genotoxic carcinogenesis, and the ability to detect mutagenicity is essential in the assessment of oil-contaminated samples (Brooks et al., 1995). The Ames test, which is based on a set of *Salmonella typhimurium* strains that revert to histidine independence upon exposure to mutagens (Kutlu et al., 2008) is one of the best known and most frequently used *in vitro* test systems to detect mutagenic effects of chemicals. The sensitivity and accuracy of this method for screening large numbers of chemicals have made it an important tool for the development of safe and useful chemicals and monitoring the environment for mutagenic threats (Greim et al., 1980). The Ames test and variations of it have been used to test the mutagenicity of polycyclic aromatic hydrocarbons in oil, water and sediment samples (Lockard et al., 1982; Sheppard et al., 1983; Vandermeulen et al., 1985).

Hydrocarbon contamination has become a problem in Peru due to the many oil incidents over the years. In Peru, 65% of the oil production occurs in the northern part of the country, and Iquitos has been the center of oil exploration and extraction in the Peruvian Amazon since 1970 (Gómez García, 1995; Oilwatch, 2001). Since then, there have been hundreds oil incidents. For instance, 78 oil spills attributed to the company Pluspetrol Peru Corporation S.A. have been reported between 2006 and 2010 (Servindi,

2010). The most recent severe incident occurred on August 2011 on the Corrientes River where an estimated 1100 barrels of oil were spilled (RPP, 2011). Another important and affected location is San José de Saramuro, which is the first station of the North Peruvian oil pipeline (854 km long) that belongs to PetroPeru S.A. Company (PetroPeru, 2000). In 2000, it was estimated that 5500 barrels were spilled in this area contaminating the Marañón River (CAAAP, 2012). Estimation of the ecological and sociological damage of these oil spills is of great importance. The Corrientes River drains into the Tigre River, which along with the Pastaza River, flows into the Marañón River. The Marañón River converges with the Ucayali River downstream of Nauta, Peru to form the Amazon River in Peru.

The Marañón River flows by the National Reserve Pacaya Samiria, the second largest protected area in Peru. The lakes in this reserve have many threatened and endangered species, such as the pink dolphin *Inia geoffrensis* and the black caiman *Melanosuchus niger* (CITES, 2006; Austermühle, 2010; Thorbjarnarson, 2010). In addition to these important species, several indigenous groups, such as the Achuar, Urarina, and Kichwa, as well as people living on the shores depend on the Amazon River and its tributaries for fishing, cooking, drinking, and other daily activities (Amazon Watch, 2010).

Due to releases of oil residues and salts released related to extraction activities, small streams show high levels of salinity that affect crops along the river and affect animals such as fish, turtles, tapirs, agoutis, and capybaras. Skin swellings and stomach pains have been reported by people in contact with or by ingestion of oil contaminated water (Goldman et al., 2007). Peru is not the only country affected since the oil industry

is spread through the entire South American continent. One of the worst freshwater incidents occurred in Venezuela where 64,000 to 120,000 barrels of crude oil were spilled in the Guarapiche River in February 2012; leaving Maturin, a nearby city, without potable water for more than a week (Carvajal and Oletta, 2012). In Bolivia, oil exposure was associated with dermic and respiratory problems (González Alonso, 2008), and in Ecuador, with spontaneous abortions, leukemia and kidney cancer (San Sebastián et al., 2002; Hurtig and San Sebastián, 2004). As oil activities and incidents continue to increase, an urgent approach for the wide range of environmental problems and adverse health effects is necessary.

The purpose of this study is to evaluate the impact of oil production activities on aquatic life in the Peruvian Amazon. This study is divided into two chapters. Chapter 2 reports LC_{50} values obtained from acute toxicity tests on a native Peruvian fish species red pacu *Piaractus brachypomus* and a standard test species, fathead minnow *Pimephales promelas*. Specific objectives were to: 1) perform acute toxicity tests on red pacu *Piaractus brachypomus* using three reference toxicants (zinc sulfate, sodium dodecyl sulfate, and Louisiana sweet crude oil) and Peruvian crude oil; and 2) perform acute toxicity tests on fathead minnows *Pimephales promelas* using Peruvian crude oil. Chapter 3 determined 1) the concentration of 16 priority PAH concentrations in oil-contaminated water and sediment from two selected sites (the Marañón River near the town of San José de Saramuro and the Corrientes River near the town of Villa Trompeteros); and 2) the EC_{50} obtained from Microtox® Acute Toxicity Test, and the mutagenicity of Peruvian crude oil, and water and sediment samples from the two selected sites.

CHAPTER 2 – ACUTE TOXICITY TESTING OF CRUDE OIL USING *PIARACTUS BRACHYPOMUS*, AND *PIMEPHALES PROMELAS*

ABSTRACT

Oil industry activities such as exploration, transportation, storage, use and disposal, as well as oil spills are sources of major contamination problems in Peru, which have significant deleterious effects on aquatic organisms. The objective of this study was to report LC_{50} values obtained from acute toxicity tests on a native Peruvian fish species, red pacu *Piaractus brachypomus*, and a designated EPA toxicity test fish, fathead minnow *Pimephales promelas*. Results showed that LC_{50} values for three reference toxicants in *Piaractus brachypomus* were: zinc sulfate = 5.74 mg/l, sodium dodecyl sulfate = 11.29 mg/l, and Louisiana sweet crude oil = 2.05 mg TPH/l. In addition, Peruvian crude oil was tested on *Piaractus brachypomus*; the LC_{50} was found to be > 4.00 mg TPH/l and the LL_{50} was found to be > 50000 mg/l; in comparison, the LC_{50} of the Peruvian crude oil in *Pimephales promelas* was 1.83 mg TPH/l, and the LL_{50} was 22920 mg/l. Results suggested that *Piaractus brachypomus* was more tolerant to the Peruvian crude oil than *Pimephales promelas*. Based on the acute toxicity tests in *Piaractus brachypomus*, the Louisiana sweet crude oil was more toxic than the Peruvian crude oil. This study is one of the few toxicity studies using Peruvian crude oil and the first one testing this fish species, but further research on other species and other toxicants related to oil contamination is necessary to assess the effects of this growing industry on the aquatic environment.

INTRODUCTION

Oil industry activities such as exploration, transportation, storage, use and disposal, as well as oil spills are sources of major contamination problems which have significant deleterious effects on aquatic organisms. Acute effects include mortality, narcosis, sublethal reproductive effects, and histopathological effects such as lesions in gill epithelium and kidney tissue inflammation. On the other hand, organisms that are exposed to oil pollution for a sustained period of time may be affected negatively; altering their growth, metabolism, and potential productivity. For example, after the *Prestige* oil spill off the coast of Galicia, Spain in 2002, thousands of birds died. Studies were made on bird blood and it was determined that yellow-legged gulls *Larus michahellis* were altered physiologically including toxic and inflammatory effects and immune-suppression (Alonso-Alvarez et al., 2007). An example of carcinogen induction from PAH contaminated areas is the elevated incidence of liver tumors found in brown bullheads *Ameiurus nebulosus* (Pinkney et al., 2004) and bottom-dwelling marine flatfish (Dunn, 1991).

Bioeffects in the environment can be examined accurately through laboratory work, including toxicity studies. Venezuelan crude oil has been widely tested on different organisms. It was found to decrease growth and chlorophyll *a* in microalgae *Tetraselmis* sp., *Chaetoceros* sp., and *Dunaliella salina* (Cortez-Mago et al., 2007). Winter flounder *Pseudopleuronectes americanus* was exposed to sediments contaminated with this crude oil causing liver hypertrophy with reduced DNA concentrations and increased lipid concentrations (Fletcher et al., 1982). In Brazil, petroleum, diesel, and gasoline were tested on marine pejerrey *Odontesthes argentinensis* larvae. The results revealed several

lesions such as hyperplasia in gills, pseudobranchs, and the esophagus (Rodrigues et al., 2010). The fish *Astyanax sp.* was exposed to water samples collected five years after an oil spill incident in Arroio Saldanha stream, southern Brazil, and a high histopathological injury index was found. Lamellar fusions and aneurysms were found in the gills, and inflammatory responses increased melanomacrophage centers in the liver (Silva et al., 2009).

Fish have been used as ecological indicators for more than 20 years in the United States and around the world (Alink et al., 2007; Vanzella et al., 2007; Baron et al., 2002; ASTM, 1993; Dunn, 1991). A typical method to measure toxicity is to perform an aquatic toxicity test to determine the concentration of a toxic material that causes 50% mortality in a population of test animals, called LC_{50} (lethal concentration) (USEPA, 2002). Different animals respond differently to the same toxin for a variety of reasons such as differences in size, anatomy, and metabolic systems. Because species vary, it is important to assess how toxic a substance is to the species of interest (Siegel, 2007).

The fish diversity in the Amazon basin is impressive and as a whole it contains more than 3000 species (USAID, 2005). Red pacu (*Piaractus brachypomus*), belonging to the family Serrasalmidae is native to the Orinoco and Amazon Rivers (Goulding, 1982), and is commercially important in the Amazon basin. While more studies on aquaculture production (Diaz and López, 1993; Rebaza et al., 2002), reproduction (Ramirez-Merlano et al., 2011), and genetic variability (Aliaga Poma, 2004; Pineda et al., 2006) have been performed in Peru, Colombia, and Bolivia, very few studies have been performed to evaluate the potential toxicity of contaminants on local and native species, and to develop appropriate assessment tools for oil-related activities.

The Amazon basin includes eight countries, and Peru represents 12% of the total area (Goulding et al., 2003). The western Amazon is a rich and still largely intact ecosystem, whose biodiversity provides services and goods of great value to the people adjacent to the river including a variety of indigenous groups. In Peru, oil exploration started in the 1920s and production peaked in the 1970s (Finer and Orta-Martínez, 2010). This economic growth has posed significant opportunities to local communities and risks to the environment. Peru is just about to enter a second oil exploration boom, and more areas are covered by proposed or active oil concessions (Finer and Orta-Martínez, 2010; Haselip, 2011). Associated oil waste effluents from Pluspetrol Peru Corporation S.A. have been discharged to small tributaries of three rivers: the Pastaza, Tigre, and Corrientes (Goldman et al., 2007). Spills and incomplete cleanups are typical in this vulnerable area, where, as recent as January, 2012, there was an oil incident where an unknown quantity of chemicals and crude oil were spilled from a corroded pipeline (Alianza Arkana, 2012). Thus, oil-related industrial activity has clearly become a threat to natural resources and the health of indigenous communities.

The purpose of this study was to report LC_{50} values obtained from acute toxicity tests on a native Peruvian fish species red pacu *Piaractus brachypomus* with comparison to a standard test species, fathead minnow *Pimephales promelas*. Specific objectives are to: 1) perform acute toxicity tests on red pacu *Piaractus brachypomus* using three reference toxicants (zinc sulfate, sodium dodecyl sulfate, and Louisiana sweet crude oil) and Peruvian crude oil; and 2) perform acute toxicity tests on fathead minnow *Pimephales promelas* using Peruvian crude oil.

METHODS

STUDY AREA

For the purpose of this study, toxicity tests on *Piaractus brachypomus*, were performed at the Laboratory of Bioactive Substances. The laboratory is part of Quistococha Biological Station owned by the Peruvian Amazon Research Institute (IIAP), and it is located on Iquitos-Nauta Road 4.5 km from Iquitos, Peru. The toxicity tests on fathead minnows *Pimephales promelas* were performed at Troy University, Troy, Alabama, U.S.A.

WATER QUALITY

Water quality parameters, such as dissolved oxygen (DO), temperature, total alkalinity, total hardness, and pH were measured before each test, and the equipment was calibrated weekly (Bringolf et al., 2007). The following equipment was used: an oximeter YSI model 55® for temperature and DO, a WTW® pH meter 330i kit for pH, and a LaMotte® freshwater test kit (model AQ-2) for total alkalinity and total hardness.

Locally available (IIAP) well water was used as dilution water and for the control of the acute toxicity tests in Iquitos, Peru and it had 32 mg/l as $CaCO_3$ of alkalinity, 24 mg/l as $CaCO_3$ of hardness, 7.1 pH, and 4.3 mg/l DO. The dilution water used in Troy, AL was aerated tap water, and it had 188 mg/l as $CaCO_3$ of alkalinity, 16 mg/l as $CaCO_3$ of hardness, 8.5 pH, and 7.5 mg/l DO.

ORGANISMS

Red pacu *Piaractus brachypomus* were provided by (IIAP) for the acute toxicity tests and were from 1 to 16 days old. Fathead minnows *Pimephales promelas* were purchased from Marinco Bioassay Laboratory and were six days old.

PREPARATION AND ANALYSIS OF WATER ACCOMMODATED FRACTION (WAF)

The American Petroleum Institute gravity (API) is an inverse measure of petroleum and water. Heavy crude oil has API gravity < 22.3° (density 920 to 1000 kg/m^3) therefore; it floats on water, while light oils' API is > 34° (Veil and Quinn, 2008). Louisiana sweet crude oil (lot #WP 681), a light oil (35.6° API) was purchased from RT Corporation, WY. The term sweet comes from the low sulfur (< 0.42%) contained in this type of petroleum (NOAA, 2010). Peruvian crude oil (for this study, was obtained from PetroPeru S.A. Company) is a heavy (20° API), sour variety with 1.2% sulfur content (Kuramoto, 2008). In order to test the oil, the water accommodated fraction (WAF) had to be prepared. The water accommodated fraction is a solution free of particles of bulk material (i.e., droplets > 1 µm diameter) derived from mixing (no vortex) test material and water (Aurand and Coelho, 1996). A 2 L borosilicate glass aspirator bottle from Thomas Scientific was used, and the sidearm was closed off with silicone tubing and a clamp. It was filled with 1 L of dilution water adding 25 g of Louisiana sweet crude oil and a second series was done for the Peruvian crude oil fraction with 1 L of dilution water adding 50 g (for *Piaractus brachypomus*), and 200 g (for *Pimephales promelas*). A stir bar was used to stir the mix on a magnetic stir plate for 22 hours in darkness. The mix was used immediately after preparation (USEPA, 2010; Singer et al., 2001).

The WAF prepared with 200 g/l of Peruvian crude oil was sent to Sitelab Corporation to be analyzed for total petroleum hydrocarbons (TPH) and total PAH concentrations. Analyses were performed on a UVF-3100 analyzer that uses ultraviolet fluorescent technology to measure hydrocarbon concentrations; the protocol is available online at http://www.stsanalytical.com/files/STS%20-%20SiteLAB%20UVF-3100.pdf (personal communication, Steve Gearson, Sitelab Corporation, May 22, 2012). The sample was weighed and methanol was added as solvent. Finally, the extract was filtered and diluted to be placed in a glass cuvette and read in the analyzer. The TPH concentration found by Hemmer et al. 2010b for Louisiana sweet crude oil (25 g/l crude oil = 2.9 mg TPH/l) was used.

ACUTE TOXICITY TESTING (STATIC)

A preliminary toxicity range-finding test was done for zinc sulfate and sodium dodecyl sulfate (SDS). Range finding is a process where the maximum concentration of toxin is determined in which the organism can survive and the minimum concentration which the organism cannot survive. Groups of three organisms were exposed to several concentrations (zinc sulfate ranged from 0.5 mg/l to 30 mg/l, and SDS ranged from 0.625 mg/l to 90 mg/l) for 24 hours. Once the approximate range to be used was determined, acute toxicity bioassays were performed for 96 hours (USEPA, 2002). The concentrations used for zinc sulfate were: 1.875 mg/l, 3.75 mg/l, 7.5 mg/l, 15 mg/l, and 30 mg/l, for SDS: 5 mg/l, 10 mg/l, 15 mg/l, 20 mg/l, and 25 mg/l, and for both oils the percentages of WAF were 6.25%, 12.5%, 25%, 50% and 100% (Appendix A). Dilution water in Iquitos, Peru for *Piaractus brachypomus* was locally available (IIAP) well water, and for *Pimephales promelas* it was aerated tap water from Troy, Alabama (ASTM,

1993; Pickering and Henderson, 1966b). New plasticware was rinsed with dilution water, while new glassware was washed with 10% hydrochloric acid and rinsed with deionized, and dilution water. All containers and equipment were flushed with dilution water before using. Borosilicate glass beakers of 250 ml were used as exposure chambers with 200 ml of respective test solutions. The temperature was kept at 28 ºC ± 1 ºC for *Piaractus brachypomus* and 25 ºC ± 1 ºC for *Pimephales promelas.* Three replicates of each concentration with 10 organisms each were run concurrently (USEPA, 2002).

Three reference toxicants were used: zinc sulfate, sodium dodecyl sulfate (SDS) purchased from Sigma-Aldrich Co. LLC., and Louisiana sweet crude oil. Peruvian crude oil available from the vicinity of Iquitos and Louisiana sweet crude oil was used as water accommodated fraction (WAF). Mortality was assessed every 24 hours, dead fish were removed (Sarikaya, 2009), and control survival was equal to or better than 90%. Results were reported as LC_{50}, defined as the concentration of a substance that causes mortality in 50% of test organisms in a specific period of time (USEPA, 2002). For Louisiana sweet crude oil, the TPH concentration found by Hemmer et al. 2010b (25 g/l crude oil = 2.9 mg TPH/l) was used to calculate the $LC_{50.}$ For Peruvian crude oil, the LC_{50} was calculated using the TPH concentration found by Sitelab Corporation in West Newbury, MA. For both crude oils the median lethal loading rate (LL_{50}), defined as the amount of the substance resulting in 50% mortality of population, was also reported (OECD, 2000).

STATISTICAL ANALYSIS

The median lethal concentration (LC_{50}) and 95% confidence intervals for each toxicant were calculated using the software Trimmed Spearman Karber (TSK) version 1.5 (Hamilton et al., 1977), available online at http://www.downloadplex.com/Scripts/Matlab/Development-Tools/Download-trimmed-spearman-karber-method-scripts_42775. Values were reported as mg/l (ppm) for zinc sulfate and sodium dodecyl sulfate, and as percentage and median lethal loadings (LL_{50}) for Louisiana sweet crude oil and Peruvian crude oil.

RESULTS

PIARACTUS BRACHYPOMUS

The LC_{50} values and 95% confidence intervals for three reference toxicants (zinc sulfate, sodium dodecyl sulfate (SDS), and Louisiana sweet crude oil), and Peruvian crude oil on *Piaractus brachypomus* are reported (Table 2.1). In addition, the LL_{50} for both crude oils are reported. Within the first 24 hours of exposure, all individuals died in the highest concentration (25 mg/l) of sodium dodecyl sulfate (SDS), and almost 50% died in the highest concentration (100%) of Louisiana sweet crude oil (Appendix A). The LC_{50} for zinc sulfate was 5.74 mg/l, and for SDS it was 11.29 mg/l. The LC_{50} found for Louisiana sweet crude oil was 70.71% using 25 g/l = 2.05 mg TPH/l and the LL_{50} was 17678 mg/l. Regarding the Peruvian crude oil, the TPH concentration of the WAF using 200 g/l of oil was found to be 16 mg/l, and it was used to calculate the LC_{50} values, while the total PAH concentration for the aquatic fraction of this mixture was 0.47 mg/l. The concentration of Peruvian crude oil (50 g/l) used to prepare the water accommodated fraction (WAF) was not enough to cause 100% mortality of organisms, not even 50%. Therefore, the actual LC_{50} value could not be calculated, but based on these data can be assumed to be > 4 mg TPH/l or > 50000 mg/l of crude oil. In general, a trend indicates that mortality percentage (%) of *Piaractus brachypomus* increased as the concentration of the toxicant increased (Figures 2.1 - 2.4). The first point in the figures represents the control; for zinc sulfate and SDS the mortality in the control was 10%, and for both oils it was about 5%.

PIMEPHALES PROMELAS

The LC_{50} for Peruvian crude oil on *Pimephales promelas* was 11.46% (1.83 mg TPH/l) with 95% confidence intervals of 6.32 – 20.79% (1.01 – 3.33 mg TPH/l). The mortality percentage (%) vs. the concentration (mg/l) is shown (Figure 2.5). Low mortality (6.5%) was observed in the highest concentration (100%) within the first 24 hours of exposure (Appendix B).

OTHER PERUVIAN FISH SPECIES

Doncella *Pseudoplatystoma fasciatum* is an important Amazonian catfish, and 7-day-old individuals were provided by (IIAP); well water was also provided as dilution water. Range finding tests were performed with two reference toxicants (zinc sulfate and sodium dodecyl sulfate) using three individuals each with the following concentrations: 0.1 mg/l, 0.3 mg/l, 1 mg/l, 3 mg/l, 10 mg/l, and 30 mg/l. A range finding test was also performed using 50 g/l of Peruvian crude oil using the following percentages of WAF: 6.25%, 12.5%, 25%, 50%, and 100%. The number of fish available was not sufficient for performing acute toxicity tests but the range finding tests showed that all individuals in 10 mg/l and 30 mg/l of zinc sulfate died. The range finding tests on SDS and the Peruvian crude oil lasted 48 hours since the individuals did not die in 24 hours with the concentrations tested. At the end of this period all individuals died in 30 mg/l of SDS and two in 10 mg/l. For the Peruvian crude oil two of the three tested individuals died in 50% and 100% (Appendix C).

Angel fish *Pterophyllum scalare* is an ornamental species, and 8-day old individuals were provided by a local breeder. Range finding tests (24 hours) were performed using 100% of water and sediment collected from the Marañón River near San

José de Saramuro, and the Corrientes River near Villa Trompeteros in Loreto, Peru (sites further explained in Chapter 3). The dilution water used for these tests was obtained from Amazon Tropical Aquarium EIRL, and the water quality was as follows: 36 mg/l as $CaCO_3$ of alkalinity, 28 mg/l as $CaCO_3$ of hardness, 7.2 pH, and 4.8 mg/l DO.

None of the five water samples from Saramuro or the six water samples from Trompeteros killed all the individuals tested in the range finding test. For sediment range finding tests all individuals were killed in Trompeteros site 5, and two in site 1 and 6 (Appendix D).

DISCUSSION

ZINC

Zinc is an essential trace constituent of natural waters and it is a required element in the metabolism of most organisms. Nevertheless, high concentrations (400 µg/l) have toxic effects on fish causing gill damage (Jones, 1938), less sexual dimorphism, liver degeneration, and muscles underdevelopment (Crandall and Goodnight, 1962). In addition, Ololade and Ogini (2009) found a decrease of leucocytes, erythrocytes and hemoglobin with increasing concentration of zinc in an African catfish, *Clarias gariepinus*. In toxicity tests, zinc is used as a reference toxicant, that is, to demonstrate acceptable laboratory performance, and to assess the sensitivity and health of organisms (USEPA, 2002).

The toxicity of zinc, as well as other heavy metals, is influenced by chemical factors including magnesium, calcium, pH, hardness, and ionic strength (USEPA, 1980). In general, heavy metals are more toxic in soft water because they are more soluble (Rathor and Khangarot, 2003). Zinc is less toxic in harder water because zinc ions' activity decreases since the ions contributing to hardness (calcium and magnesium) compete with zinc for binding sites and uptake in biological tissue (Pyle et al., 2002; Kim et al., 2001). In previous studies using about the same hardness (24 mg/l as $CaCO_3$) with different fish species, the LC_{50} values for zinc sulfate ranged from 0.6 mg/l to 6.4 mg/l (Table 2.2). Ebrahimpour et al. (2010) tested different water hardnesses, finding that zinc toxicity increased with softer water. However, toxicity varies among individuals, species, and larger phylogenetic groups (Kim et al., 2001). For instance, a toxicity study on mottled sculpin *Cottus bairdi* suggested that this species has the lowest acute toxicity

to zinc (0.156 mg/L) than any other fish tested to date (Woodling et al., 2002). Similar hardness (20 mg/l as CaCO3) to the one in the present study was used by Pickering and Henderson, 1966a, who reported similar LC_{50} values for bluegill *Lepomis macrochirus* (5.82 mg/l), and goldfish *Carassius auratus* (6.4 mg/l) compared to *Piaractus brachypomus*. Pickering and Henderson (1966a) also found that the LC_{50} for guppy *Poecilia reticulata* was 1.27 mg/l, and for fathead minnow *Pimephales promelas* it was 0.78 – 0.96 mg/l, suggesting that these species are more sensitive to zinc toxicity.

SODIUM DODECYL SULFATE

Sodium dodecyl (lauryl) sulfate is an organic compound used as a reference toxicant (USEPA, 2002). The 96-h LC_{50} for red pacu *Piaractus brachypomus* reported herein is 11.29 mg/l, which is slightly higher than the value reported for other fish species such as the inland silverside *Menidia beryllina* (9.5 mg/l; Hemmer et al., 2010a), and fathead minnow *Pimephales promelas* (8.6 mg/l; USEPA, 2002), but less than the killifish *Cynopoecilus melanotaenia* (14.9 mg/l; Arenzon et al., 2003) (Table 2.3).

CRUDE OIL

In the current study, the LC_{50} for Peruvian crude oil on red pacu *Piaractus brachypomus* was higher than the LC_{50} value found for fathead minnow *Pimephales promelas*, suggesting that the Peruvian species might be less sensitive to this crude oil. *Piaractus brachypomus* was tested with two crude oils, and the LC_{50} for the Louisiana sweet crude oil was lower than the Peruvian crude oil, indicating higher toxicity. This was expected since the two crude oils had different density (API), therefore, different properties. The Peruvian crude oil was heavy, which USEPA (2011a) describes as

viscous, black, and having low toxicity. The Louisiana sweet crude oil was light, described as highly fluid and toxic.

Different crude oils tested on fish species are compared with the Peruvian and Louisiana crude oil in the present study. Previous studies range from the Prudhoe Bay crude oil (> 0.5 mg TPH/l) to the Arabian medium crude oil (> 14.5 mg TPH/l) (Table 2.4). However, comparisons on effects of crude oil WAF are difficult since the composition of hydrocarbons in the oils vary depending on their density and origin (Neff et al., 2000). Other factors influencing the widely different results is the preparation method of the WAF between studies, which include room temperature, mixing energy, settling period, and the tolerance to crude oil of the species tested (Singer et al., 2001). Furthermore, toxicity of crude oil seems to be lower in marine species compared to freshwater probably due to hydrocarbon solubility and lower bioaccumulation in fish when salinity is increased (Ramachandran et al., 2006). Inland silverside *Menidia beryllina* is an estuarine and EPA approved marine species commonly used in toxicity testing (Hemmer et al., 2010a). Several crude oils have been tested on this species such as Arabian medium (LC_{50} = > 14.5 mg TPH/l), Alaska North Slope (LC_{50} = 0.35 mg TPH/l), and Kuwait (LC_{50} = > 1.32 mg TPH/l) showing the high variability of LC_{50} values using different crude oils.

Crude oil contains poorly soluble components that are influenced by changes in temperature or chemical changes due to weathering. Therefore, it is recommended to report the results of materials with low solubility components as the median lethal loading rate (LL_{50}), defined as the amount of the substance resulting in 50% mortality of the population (Peterson, 1994). The loading rate used for Peruvian crude oil on red pacu

Piaractus brachypomus (50 g/l) was not enough to kill 50% of the test organisms; therefore neither the LC_{50} nor LL_{50} could be calculated. However, the result was reported as > 50000 mg/l, almost twice as high as the LL_{50} for Kuwait and North Sea Forties crude oils tested on *Menidia beryllina* and *Scophthalmus maximus*, respectively (Clark et al. 2001). In the literature found, Alaska North Slope had the lowest LL_{50} (3520 mg/l) for inland silverside *Menidia beryllina*, suggesting high toxicity. Indeed, Brand et al. (2001) found that WAF from the Alaskan crude oil caused stress and morphologic lesions in gills, hepatic and kidney tissues on pink salmon fry *Oncorhynchus gorbuscha*.

CONCLUSIONS

This study reported LC_{50} values on a native fish species, red pacu *Piaractus brachypomus*, for three reference toxicants, zinc sulfate = 5.74 mg/l, sodium dodecyl sulfate = 11.29 mg/l, and Louisiana sweet crude oil = 2.05 mg TPH/l. When testing crude oil, it is recommended to report the LL_{50} to better compare the results to other studies. Peruvian crude oil was tested on *Piaractus brachypomus*, and the LC_{50} was found to be > 4.00 mg TPH/l, and the LL_{50} was found to be > 50000 mg/l. The same Peruvian crude oil was tested on fathead minnow *Pimephales promelas* and the LC_{50} was 1.83 mg TPH/l, while the LL_{50} was found to be 22920 mg/l.

Piaractus brachypomus was found to be more tolerant to the Peruvian crude oil than *Pimephales promelas*. Based on the acute toxicity tests in *Piaractus brachypomus*, the Louisiana sweet crude oil was more toxic than the Peruvian crude oil, due to the properties of the oils since the Peruvian crude oil is considered heavy and less toxic compared to light crude oils.

Bioassays are an important tool used to provide background information for risk assessment of chemicals. This study is one of the few toxicity studies using Peruvian crude oil and the first one using this fish species, which showed potential as a test organism in toxicity testing. However, further research on other species and other toxicants such as lead, cadmium and mercury, related to oil contamination is necessary to assess the effects of this growing industry on the aquatic environment.

Table 2.1. Median lethal concentrations (LC_{50}) and 95% confidence intervals for 96 hour toxicity tests on *Piaractus brachypomus.* Also median lethal loadings (LL_{50}) only for crude oils. Note: WAF = Water accommodated fraction, TPH = Total petroleum hydrocarbons, N/A = not available.

Toxicant	96 h - LC_{50}	96 h – LL_{50} (mg/l)
Zinc sulfate (mg/l)	5.74 (3.62 - 9.08)	N/A
Sodium dodecyl sulfate (SDS) (mg/l)	11.29 (8.36 - 15.26)	N/A
Louisiana sweet crude oil (WAF) (mg TPH/l)	2.05 (1.81 - 2.30)	17678
Peruvian crude oil (WAF) (mg TPH/l)	> 4.00 (N/A)	> 50000

Table 2.2. Median lethal concentrations (LC_{50}) for zinc toxicity tests on different fish species.

Fish species name	Hardness	96- h LC_{50} (mg/l)	Reference
Mottled sculpin *Cottus bairdi*	48.6	0.156	Woodling et al. (2002)
Rainbow trout *Oncorhynchus mykiss*	30	0.2 - 0.83	Goettl et al. (1972)
Atlantic salmon *Salmo salar*	20	0.6	Sprague (1964)
Fathead minnow *Pimephales promelas*	20	0.78 - 0.96	Pickering and Henderson (1966a)
Fathead minnow *Pimephales promelas*	360	33.4	Pickering and Henderson (1966a)
Fathead minnow *Pimephales promelas*	203	13	Brungs (1969)
Fathead minnow *Pimephales promelas*	203	8.4	Brungs (1969)
Guppy *Poecilia reticulata*	20	1.27	Pickering and Henderson (1966a)
Bluegill *Lepomis macrochirus*	20	5.82	Pickering and Henderson (1966a)
Goldfish *Carassius auratus*	20	6.4	Pickering and Henderson (1966a)
Siah mahi *Capoeta fusca*	40	13.7	Ebrahimpour et al. (2010)
African catfish *Clarias gariepinus*	193.3	36.7	Ololade and Ogini (2009)
Red pacu *Piaractus brachypomus*	24	5.74	Present study

Table 2.3. Median lethal concentrations (LC_{50}) for sodium dodecyl sulfate (SDS) toxicity tests on different fish species.

Species	96- h LC_{50} (mg/l)	Reference
Inland silverside *Menidia beryllina*	9.5	Hemmer et al. (2010a)
Fathead minnow *Pimephales promelas*	8.6	USEPA (2002a)
Killifish *Cynopoecilus melanotaenia*	14.9	Arenzon et al. (2003)
Red pacu *Piaractus brachypomus*	11.29	Present study

Table 2.4. Median lethal concentrations (LC_{50}) and median lethal loadings (LL_{50}) for different crude oil toxicity tests on different fish species. Note: TPH = Total petroleum hydrocarbons, N/A = not available.

Fish species name	Crude oil type	96- h LC_{50} (mg TPH/l)	96- h LL_{50} (mg/l)	Reference
Inland silverside *Menidia beryllina*	Arabian medium	> 14.5	N/A	Fuller and Bonner (2001)
Sheepshead minnow *Cyprinodon variegatus*	Arabian medium	> 6.1	N/A	Fuller and Bonner (2001)
Inland silverside *Menidia beryllina*	Alaska North Slope	0.35	3520	Rhoton et al. (2001)
Inland silverside *Menidia beryllina*	Prudhoe Bay	> 0.5	> 8152	Rhoton et al. (2001)
Inland silverside *Menidia beryllina*	Louisiana sweet	3.5	N/A	Hemmer et al. (2010b)
Inland silverside *Menidia beryllina*	Kuwait	> 1.32	> 25000	Clark et al. (2001)
Turbot *Scophthalmus maximus*	North Sea Forties	> 1.33	> 23471	Clark et al. (2001)
Crimson-spotted rainbowfish *Melanotaenia splendida fluviatilus*	Australian	1.28	N/A	Pollino and Holdway (2002)
Rainbow trout *Oncorhynchus mykiss*	Marathon petroleum	N/A	0.021	American Petroleum Institute (2003)
Red pacu *Piaractus brachypomus*	Louisiana sweet	2.05	17700	Present study
Red pacu *Piaractus brachypomus*	Peruvian	> 4.00	> 50000	Present study
Fathead minnow *Pimephales promelas*	Peruvian	1.83	22920	Present study

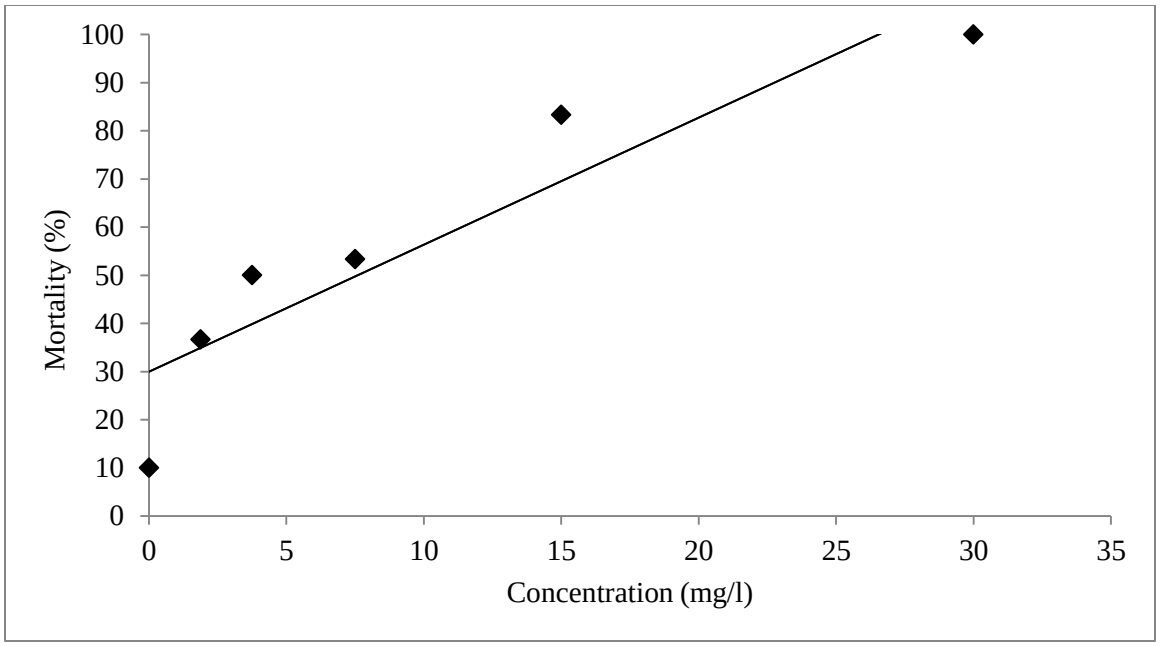

Figure 2.1. Mortality percentage (%) vs. concentration (mg/l) of zinc sulfate on red pacu *Piaractus brachypomus.*

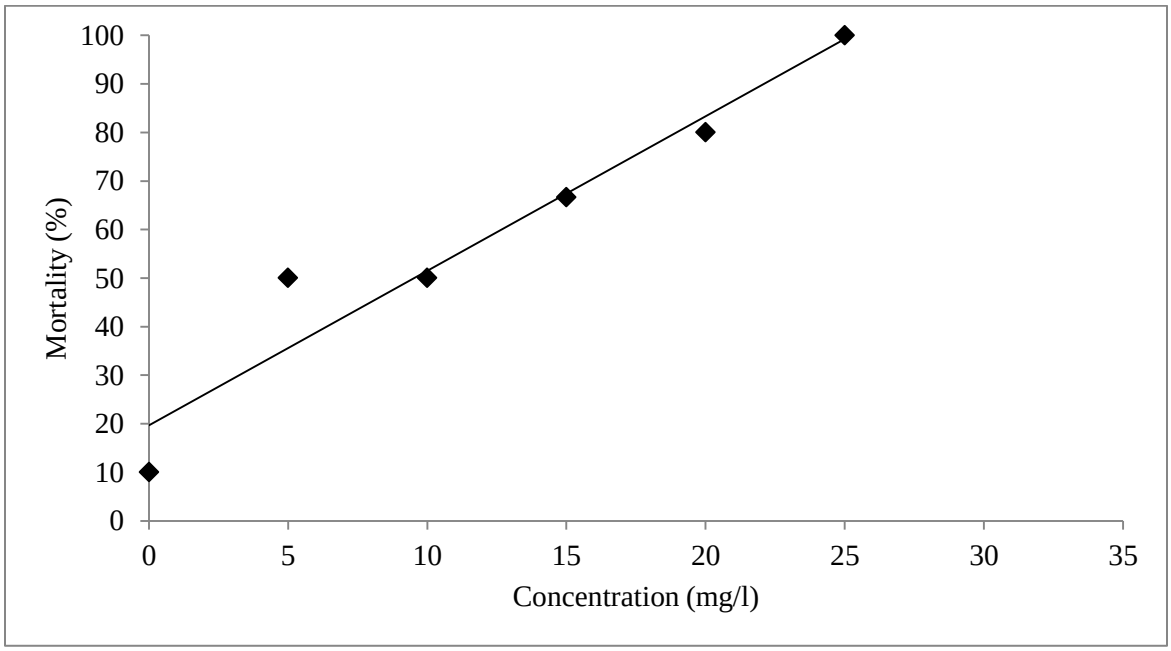

Figure 2.2. Mortality percentage (%) vs. concentration (mg/l) of sodium dodecyl sulfate (SDS) on red pacu *Piaractus brachypomus.*

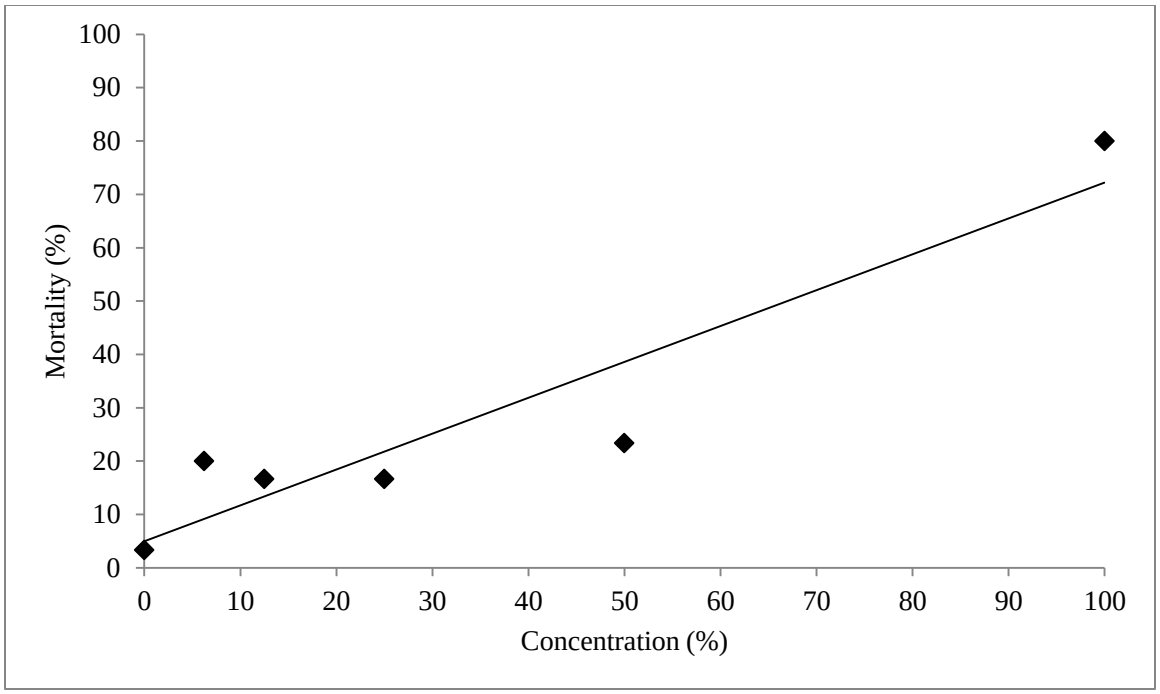

Figure 2.3. Mortality percentage (%) vs. concentration (%) of water accommodated fraction (WAF) using 25 g/l of Louisiana sweet crude oil on red pacu *Piaractus brachypomus.*

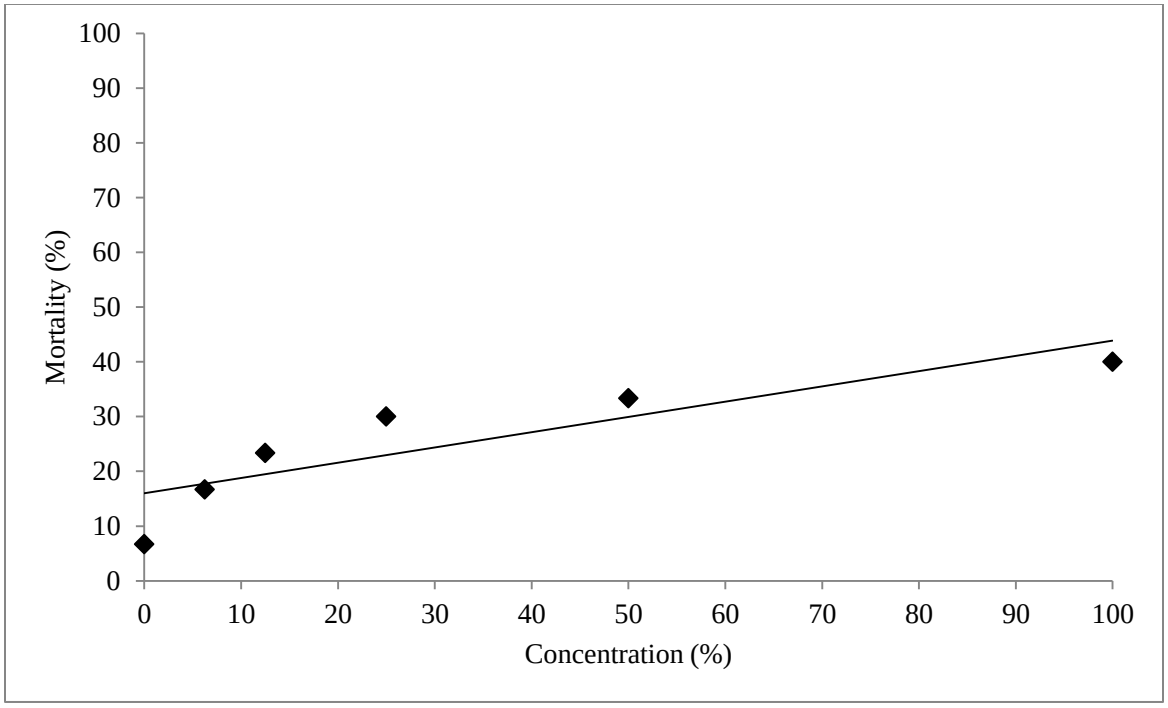

Figure 2.4. Mortality percentage (%) vs. concentration (%) of water accommodated fraction (WAF) using 50 g/l of Peruvian crude oil on red pacu *Piaractus brachypomus.*

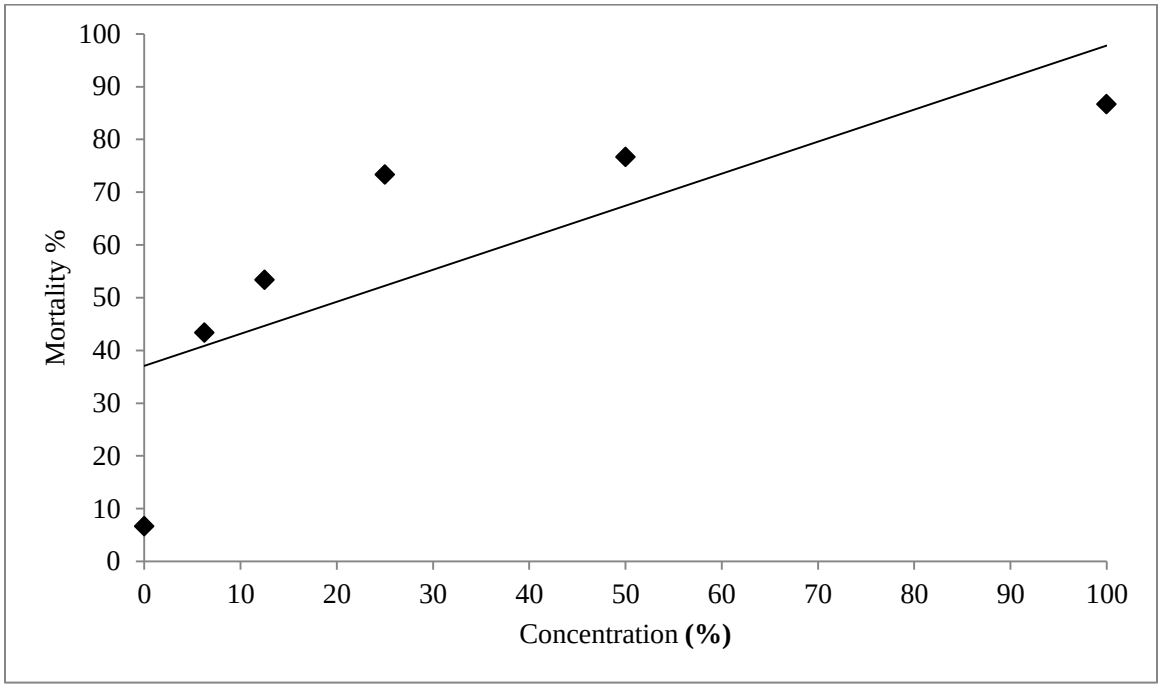

Figure 2.5. Mortality percentage (%) vs. concentration (%) of water accommodated fraction (WAF) using 200 g/l of Peruvian crude oil on fathead minnows *Pimephales promelas.*

CHAPTER 3 – POLYCYCLIC AROMATIC HYDROCARBON CONCENTRATIONS, MUTAGENICITY, AND MICROTOX ACUTE TOXICITY TESTING OF PERUVIAN CRUDE OIL AND OIL-CONTAMINATED WATER AND SEDIMENT

ABSTRACT

The oil industry is a major source of contamination in Peru, and wastewater and sediments containing oil includes harmful substances which may have acute and chronic effects. This study determined PAH concentrations, mutagenicity, and Microtox EC_{50} values of Peruvian crude oil, and water and sediment from the vicinity of San José de Saramuro on the Marañón River and Villa Trompeteros on the Corrientes River in Loreto, Peru. Gas chromatography/mass spectrophotometry was used for PAH concentrations, the Muta-ChromoPlate™ was used for mutagenicity using strain TA98 and TA100, and the Microtox® Acute Toxicity Test was used to determine the EC_{50}. The highest total PAH concentration in both areas was found in water (Saramuro = 210.15 µg/ml, Trompeteros = 204.66 µg/ml). All water samples tested from Saramuro and Trompeteros sites, and one sediment sample from Trompeteros, were found to be mutagenic ($P < 0.001$). A sediment sample in Saramuro was found to have a measurable toxicity (Microtox EC_{50} = 335.1 mg/l), and in Trompeteros the EC_{50} in water and sediment ranged from 25.67 to 133.86 mg/l. Peruvian crude oil was mutagenic using strain TA98 and S9 enzyme, and the EC_{50} was 17.18 mg/l. The two areas sampled had very high PAH concentrations that are most likely associated with oil activities, but the acute toxic effects were beyond the level of detection. However, since most of the samples were mutagenic, it is thought that the DNA structure in organisms could be affected, suggesting the need of further and more extensive research.

INTRODUCTION

There is concern about polycyclic aromatic hydrocarbons (PAHs) contained in oil, due to their pathological and carcinogenic properties (van Hattum and Montañés, 1999; Niimi and Palazzo, 1986). The oil industry is spread throughout the entire South American continent having negative impacts on the population. In Bolivia, oil exposure was associated with dermic and respiratory problems (González Alonso, 2008), and in Ecuador, with abortions, leukemia and kidney cancer (San Sebastián et al., 2002; Hurtig and San Sebastián, 2004). In Peru, skin swellings and stomach pains have been reported by people in contact or by ingestion of oil contaminated water and food (Goldman et al., 2007). However, few Peruvian studies have focused on PAHs contained in water and sediment contaminated from oil activities and incidents.

Toxicity bioassays are a prescreening tool for the chemical assessment of polluted samples (De Zwart and Slooff, 1983). The Microtox system is an assay based on inhibition of light emitted by the bioluminescent marine bacteria *Vibrio fischeri*, formerly known as *Photobacterium phosphoreum*. Microtox® has been successfully used as a screening system to detect the relative toxicity of fungi, such as *Aspergillus fumigatus* (Alba et al., 2009), pesticides (Ruiz et al., 1997), industrial waste (Hao et al., 1996), water-soluble crude oil fractions (Ziolli and Jardim, 2002), and oil contaminated soil and sediment (Loureiro et al., 2005, Blaise et al., 2004).

Wastewater containing oil contains harmful substances, including those with genotoxic effects that are described as any process that affects DNA structure (Bohne and Cathomen, 2008). Genotoxicity studies in Ecuador, on the Amazonian population close to crude oil extraction zones, have shown DNA damage such as type B nuclei fragmentation and chromosomal aberrations (Paz-y-Miño et al., 2012). A distillate from

Venezuelan crude oil was found to increase DNA adduct formation in rat liver (Nagy et al., 2004). Numerous spills and leakages involving petroleum have occurred in Brazilian rivers and genotoxicity assays have also been performed. For instance, chromosomal aberration assays on *Allium cepa* exposed to petroleum polluted water showed breaks in chromosomes and changes in chromosome number (Leme et al., 2008). Nuclear degeneration and bi-nucleated hepatocytes have been found in marine pejerrey *Odontesthes argentinensis* exposed to water soluble fractions of diesel and gasoline (Rodrigues et al., 2010).

Mutagenicity is a critical step in genotoxic carcinogenesis development (Brooks et al., 1995), and several PAHs have been found to be carcinogenic or possible human carcinogens (IARC, 1983). Different strains designated to determine mutagens activate carcinogenic PAHs, these tester strains detect different mutational events, either frameshift mutation (strain TA98, TA97) or base pair substitution (strain TA100, TA102) (Maron and Ames, 1983). A very common test to identify environmental mutagens and potential carcinogens is the Muta-ChromoPlate^{TM} test, which uses a mutant strain of *Salmonella typhimurium* that carries mutation in the operon coding for histidine biosynthesis (Zeiger and Mortelmans, 1999).

Mutagenicity and acute toxicity results are used as scientific basis to determine regulatory uses and research needs in risk assessment of potential contamination problems. This study determined PAH concentrations, mutagenicity, and EC_{50} values of Peruvian crude oil and water and sediment from two contaminated areas in proximity to oil extraction and transportation in Loreto, Peru.

METHODS

STUDY AREA

Water and sediment samples were collected from two areas near oil-related activities, both about 200 km from Iquitos, the main Amazonian city in the Loreto Region, Peru (Figure 3.1). Five sites were selected on the Marañón River near the town of San José de Saramuro, southeast of Iquitos (Figure 3.2, Table 3.1 - 3.2). The Marañón River originates in the Peruvian Andes and its width varies from 800 to 2600 m (about 500 m at the sampling site). The bottom is mainly sand, lime and clay, and depth varies seasonally from 3 m in August to 8 m in April (IIAP, 2002). San José de Saramuro (~2000 inhabitants) is the first station of the North Peruvian oil pipeline (854 km long) that belongs to PetroPeru S.A. Company. The pipeline goes to the west across the Andes to the north coast of Peru, finally arriving at Sechura Bay, on the Pacific coast (PetroPeru, 2000).

Six sites were selected on the Corrientes River near the town of Villa Trompeteros, east of Iquitos (Figure 3.2, Table 3.3 - 3.4). The Corrientes River has its origins in the Ecuadorian highlands and it was about 100 m wide at the sampling site. Both the Marañón and Corrientes River have white water (i.e., high concentrations of sediments on the surface, total suspended solids = 109 mg/L and high conductivity > 150 µS) (Barthem et al, 2003). The Corrientes River drains to the Tigre River, which drains to the Marañón River, a main tributary of the Amazon River. Villa Trompeteros is the nearest town to the oil activities complex called ‘Block 8’ that belongs to the Argentinian oil and gas company, Pluspetrol Peru Corporation S.A. Block 8 contains 29 native communities and 3900 inhabitants (Ministerio de Energía y Minas, 2009).

WATER QUALITY

Water quality parameters: dissolved oxygen (DO), temperature, total alkalinity, total hardness, and pH were determined at the point of collection of the field samples. An oximeter YSI model 55® was be used for temperature and DO, a Xylem, Inc. pH meter 330i kit was used for pH, and a LaMotte® freshwater test kit (model AQ-2) was used for total alkalinity, and total hardness (Appendix C).

WATER SAMPLING AND ANALYSIS

Certified low-density polyethylene (LDPE) collapsible cubitainers of 1 L each (VWR International, LLC) were used for water sampling, and rinsed with native water before use. Grab samples were collected at ~15 cm depth. All sample cubitainers were completely filled under water leaving no air space between the sample and the lid. Samples were taken to the laboratory on ice and stored in the dark at 0-6 ºC until PAH analysis, within three weeks (USEPA, 2002).

Sample extracts were prepared using EPA method 550 (USEPA, 1990). A liter of the sample was poured into a separatory funnel. Methylene chloride (60 ml) was added, and the funnel was shaken for two minutes with periodic venting. The organic layer was allowed to separate from the water for 10 minutes, and the methylene chloride extract was collected in an Erlenmeyer flask. This procedure was repeated two more times. A Kuderna-Danish (K-D) concentrator was assembled by attaching a 500 ml evaporative flask to a 10 ml concentrator tube. All the extract was poured through a solvent-rinsed drying column with 10 cm of anhydrous sodium sulfate and collected in the K-D concentrator. Two boiling chips were added to the evaporative flask and attached to a three-ball Snyder column. The K-D apparatus was placed on a hot water bath for 20 minutes. When the volume of liquid reached 0.5 ml, it was transferred and stored in a

Teflon-sealed screw-cap borosilicate vial wrapped with aluminum foil to protect it from light, and stored at 4 °C.

SEDIMENT SAMPLING AND ANALYSIS

A stainless steel bottom sampling Ekman dredge (Code 1097, LaMotte®) was used for collection of sediments. Prior to sampling and between sites, the dredge, scoop, bucket, and glass containers were washed with phosphate-free detergent, and then rinsed with tap water and deionized water, with a final methanol rinse. After this, the equipment was wrapped in aluminum foil and kept in a plastic container until use. Once in the field, all equipment was rinsed with native water prior to use. Each bottom sample was mixed and placed in a 1 L glass jar sealed with a lid and Parafilm®. Samples were taken to the laboratory on ice and stored in the dark at 0-6 ºC until use, within three weeks (Arizzi Novelli et al., 2006; Shelton and Capel, 1994). Sediment samples from Trompeteros sites 2 and 3 (inside and directly outside the Trompeterillo stream) could not be collected in the Corrientes River, since oil company security staff did not allow sediment collection. Sediment sampling in Trompeteros sites 5 and 6 had to be done with a stainless steel scoop since the shoreline was too shallow, and the current was too fast for use of the Ekman dredge.

Sample extracts were prepared using the Northwest Total Petroleum Hydrocarbon Identification analytical method (NWTPH-HCID) (Oregon Department of Environmental Quality, 1996). Moisture content of the samples was determined by weighing 5 g of the mixed sample into a tared crucible. The sample and crucible were dried overnight at 105 °C. They were cooled at room temperature and weighed again. The percentage of solids was calculated as follows:

$$\% = \frac{\text{Weight of dry sample}}{\text{Weight of wet sample}} * 100$$

Ten grams of sediment was weighed into a volatile organic analysis (VOA) vial, 5 g of anhydrous sodium sulfate, and 10 ml of methylene chloride were added. Vials were placed in a sonic bath for five minutes. Extracts were poured through a solvent-rinsed drying column with 10 cm of anhydrous sodium sulfate, and stored in a Teflon-sealed screw-cap vial wrapped with aluminum foil to protect it from light, and stored at 4 °C.

ANALYSIS OF POLYCYCLIC AROMATIC HYDROCARBONS

Polycyclic aromatic hydrocarbons, from water and sediment samples, were analyzed using a gas chromatograph/mass spectrophotometer (GC/MS) VARIAN 450 following EPA method 8270C (USEPA, 1996b). Sample extracts (1 µl) were injected into the gas chromatograph with a narrow-bore fused-silica capillary column. The column DB5-MS (30 m x 0.25 mm diameter, 0.25 µm film thickness) separated the analytes that were detected with the mass spectrometer. The temperature program was: 80 °C for five minutes, increased to 290 °C at three °C/minute, and held for 30 minutes. Quantification was done using the EPA 610-N PAH mix from the company Sigma-Aldrich to determine 16 polycyclic aromatic hydrocarbons (PAH) (Scoggins et al., 2007). The mix includes naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benz[a]anthracene, chrysene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, dibenzo[a,h]anthracene, indeno[1,2,3-cd]pyrene, and benzo[ghi]perylene (organized by molecular weight).

PREPARATION OF WATER ACCOMMODATED FRACTION (WAF)

Peruvian crude oil (for this study, was obtained from PetroPeru S.A.) is a heavy, sour variety with 1.2% sulfur content and 20° API (Kuramoto, 2008). The American Petroleum Institute gravity (API) is an inverse measure of petroleum and water. Heavy crude oil has an API gravity below 22.3° (density 920 to 1000 kg/m^3), therefore; it floats on water (Veil and Quinn, 2008). In order to test the oil, the water accommodated fraction (WAF) had to be prepared. The water accommodated fraction is a solution free of particles of bulk material (i.e., droplets $\geq$ 1 μm diameter) derived from mixing (no vortex) test material and water (Aurand and Coelho, 1996). A 2-L borosilicate glass aspirator bottle from Thomas Scientific, Inc., was used, with the sidearm closed off with silicone tubing and a clamp. The bottle was filled with 1 L of dilution water adding 200 g of Peruvian crude oil, leaving a 20% headspace above the liquid. A stir bar was used to stir the mix on a magnetic stir plate for 22 hours in darkness. The mix was used immediately after preparation (USEPA, 2010; Singer et al., 2001).

MUTA-CHROMOPLATE™

The water and sediment analyses for the mutagenicity tests were performed at the Laboratory of Bioactive Substances, part of the Quistococha Biological Station (Instituto de Investigaciones de la Amazonía Peruana), located on the Iquitos-Nauta Road 4.5 km from Iquitos, Peru.

The Muta-chromoplate™ kit is a liquid culture assay based on the Ames test, and it uses *Salmonella typhimurium* strains that revert to the amino acid histidine independence upon exposure to mutagens. Materials and chemicals were purchased from Environmental Biodetection Products Incorporation (EBPI). The test was done using the

Muta-ChromoPlate™ Basic kit protocol (EBPI, 2005). All samples (water, sediment and crude oil) were prepared in duplicate, using *Salmonella typhimurium* test strain TA98, which detects frameshift mutations, and TA100, which detects base pair substitutions. However, not all samples were tested due to lack of reagents and plates.

Mutagenicity of water and sediment samples was tested by a liquid culture of the fluctuation test without metabolic activation (S9 enzyme). While the water accommodated fraction (WAF) prepared with Peruvian crude oil was tested with and without metabolic activation (S9 enzyme). This enzyme extract is derived from rat crude liver and can genetically active genotoxic entities (EBPI, 2005).

The reaction mixture was prepared mixing 21.62 ml Davis Mingioli medium, 4.75 ml D-glucose, 2.38 ml bromocresol purple, 1.19 ml D-biotin, and 0.06 ml L-histidine. About 30 ml of the aqueous sample was filter sterilized using a 0.22 µm sterile filter. For sediment samples, 0.1 g of the sample was mixed with 0.5 ml dimethyl sulfoxide (DMSO) and 17.5 ml of distilled water, and then sterile filtered as was the aqueous sample. Samples were mixed with water, reaction mixture, and bacterial suspension (TA98 and TA100) from the culture grown overnight (Table 3.3). Contents of each tube, 200 µl aliquots of the mixture were dispensed into each well of a 96-well microtitration plate. The plate was covered with a lid and sealed in an airtight plastic bag to prevent evaporation. Two negative controls (backgrounds samples), one for TA98 and another for TA100, which contained the reaction mixture, water, and the bacteria, were used in order to make comparisons with the treatment plate. A blank, and positive controls containing sodium azide (NaN_3) and 2-nitrofluorine (2-NF), two known direct-acting mutagens, were also used (Table 3.3). For the Peruvian crude oil, the S9 enzyme (a crude rat liver

extract to activate metabolism) was added to the treatment plates, and a positive control using 2-amino anthracene (2-AA, requires enzymatic activation) was used (Table 3.4). Plates were incubated at 37 ^{o}C for five days. After the incubation period, plates were scored visually by counting yellow or turbid wells as positives and purple wells were scored as negatives.

MICROTOX®

The Microtox® Acute Toxicity Test of water, sediment samples, and Peruvian crude oil were performed at the Troy University Environmental Laboratory, Alabama, U.S.A. The Microtox® assay exposes the marine luminescent bacteria *Vibrio fischeri* to osmotically adjusted, serially diluted sample treatments while measuring the increase or decrease in light output by the test organisms relative to a reference (control) sample. The toxicity is expressed in terms of EC_{50} (half maximal effective concentration) (Doherty, 2001). The benefit of Microtox® is that it provides an informative toxicity measure of single or multiple hazardous pollutants in the sampled media (Berglind et al., 2010).

The Microtox® bacterial assay was used to determine 5-minute EC_{50} values using the Microbics Corporation (1992) protocol and a Microbics M500 toxicity analyzer. Freeze-dried bacteria (available from Azur Environmental, previously Microbics Corporation) were rehydrated immediately prior to use in testing (Doherty, 2001). Phenol was used as a standard, and the sample of Peruvian crude oil was done in triplicate. Sediment samples collected from Saramuro and Trompeteros were centrifuged for an hour with no water added to obtain clear supernatant. Initial light readings for cuvettes containing reconstituted bacteria were measured on the analyzer. Two-fold

serial dilutions of the sample were made to produce eight exposure concentrations. On computer commands, the light levels at five minutes were reread.

DATA ANALYSIS

The mutagenicity of the sample was determined by comparing the number of wells positive in the background plate to the number of wells positive in the treatment plate (Zeiger and Mortelmans, 1999), and statistical differences were determined using the table for analysis of results of fluctuation tests developed by Gilbert (1980) (EBPI, 2005). The Mutagenic Ratio (MR) was determined as the number of histidine revertants in a test plate divided by the number of spontaneous revertants of the negative control (Lupi et al., 2009). The EC_{50} (effective concentration causing 50% light loss) was determined by calculating the control ratio/gamma (CR/gamma) for all exposure concentrations, then determining the concentration at which the ratio of the light lost to the light remaining equals one. Toxicity increases when EC_{50} decreases.

RESULTS

POLYCYCLIC AROMATIC HYDROCARBON CONCENTRATIONS

This study analyzed the concentration of 16 priority PAHs and the total PAH concentration in water and sediment samples from San José de Saramuro (S1 – S5) on the Marañón River and Villa Trompeteros (T1 – T6) on the Corrientes River. Results are averages of three replicates and the standard deviation in some sites is higher than the average since some replicates had concentrations that were below detection limits. Each of the 16 priority PAHs were detected in at least one of the sites. The PAH concentrations in water samples from both sites ranged from 7.54 to 210.15 µg/ml and sediment samples varied from 2.19 to 70.41 µg/ml. These concentrations are within the range of other studies done in South America (Table 3.9).

SAN JOSÉ DE SARAMURO. No PAHs were detected in water at S1 and the total PAH concentration in the rest of the sites ranged from 7.54 µg/ml at S5 to 210.15 µg/ml at S3 (Table 3.5). The PAH that contributed the most for the total concentration in water from S2, S3 and S4 was dibenzo[a,h]anthracene, and from S5 was benzo[a]pyrene. Sediment concentrations ranged from 2.19 µg/ml at S2 to 70.41 µg/ml at S5. Benzo[a]pyrene (BaP) was detected in all sediment samples with the highest concentration at S5, and at S1 and S2, it was the only PAH detected (Figure 3.4). The PAHs with low molecular weight are naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, and fluoranthene, and contributed around 12% (water) and 23% (sediment) to the total PAH concentration in the area (Figure 3.4 – 3.5).

VILLA TROMPETEROS. No PAHs were detected in water at T1, T2, T3, and T5, while fluoranthene was the only hydrocarbon detected at T4 with 20.71 µg/ml. At

T6, all sixteen priority PAHs were detected with a total PAH concentration of 204.66 µg/ml, from which anthracene contributed the most with 70.08 µg/ml (Table 3.6 and Figure 3.6). On average, the PAHs with low molecular weight contributed the most in this sample, around 64%. The PAHs detected in sediment samples were fluoranthene, pyrene, benzo[k]fluoranthene, benzo[a]pyrene, and dibenzo[a,h]anthracene. The total PAH concentration ranged from 3.59 µg/ml at T1 to 67.33 µg/ml at T4. Benzo[a]pyrene was detected in all sediment samples, the highest concentration was found at T4, and it contributed the most at T4, T5 and T6 (Figure 3.7).

MUTA-CHROMOPLATE™

The mutagenic profiles of controls and samples in the two study areas are shown (Table 3.7). The revertant colonies in negative-control plates were six for TA98 and 10 for TA100. The mutagenicity ratio (MR: number of histidine revertants in a test plate divided by the number of spontaneous revertants of the negative control) was higher for TA98 in all the samples compared to TA100, except sediment samples from T1, T5 and T6. The three water samples tested from San José de Saramuro and the two water samples from Villa Trompeteros were found to be mutagenic ($P < 0.001$) with strain TA98 and TA100. One of four sediment samples from Villa Trompeteros was found to be mutagenic ($P < 0.001$) for both strains. None of the water and sediment samples was tested with S9 enzyme (metabolic activation).

The mutagenic profiles of controls and WAF using Peruvian crude oil are shown (Table 3.8). Strains TA98 and TA100 were both tested with and without S9 enzyme (metabolic activation). The MR varied from 0.13 to 1.46. Peruvian crude oil was found to be mutagenic ($P < 0.001$) in bacterial strain TA98 containing S9 enzyme.

MICROTOX®

The 5-minute EC_{50} values for 11 water samples and nine sediment samples are shown (Table 3.7). One water sample of 11 had an EC_{50} of 133.86 mg/l (T4), and three sediment samples of nine samples were S4 = 335.10 mg/l, T4 = 25.67 mg/l, T5 = 69.38 mg/l. The EC_{50} for WAF using Peruvian crude oil was 17.18 mg/l, the average of four replicates (Table 3.8). The EC_{50} in the Peruvian crude oil was lower than the water and sediment samples, suggesting higher toxicity.

DISCUSSION

POLYCYCLIC AROMATIC HYDROCARBONS

The present study analyzed water and sediment samples from San José de Saramuro on the Marañón River and Villa Trompeteros on the Corrientes River. The highest Σ PAH concentration in water from Saramuro was 210.15 µg/ml at S3, sampled 100 m downstream from the main pipeline in the area. The sediment Σ PAH concentration at S3 (33.36 µg/ml) was high, but not as high as at S5 (70.41 µg/ml) located 150 m downstream from a second pipeline. These results suggest that contaminants (PAHs) in Saramuro are carried downstream and bind to the sediment.

The persistence of PAHs is related directly to their molecular weight. The PAHs with two and three rings have low molecular weight; these are naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, and fluoranthene. Most of these low molecular PAHs were present in water from S3 (also highest Σ PAH concentration), which was the nearest collection site from the main pipeline in the area. Meanwhile, the ones with more rings have greater molecular weight; these are pyrene, benz[a]anthracene, chrysene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, dibenzo[a,h]anthracene, indeno[1,2,3-cd]pyrene, and benzo[ghi]perylene (ATSDR, 1995). All of these were found in either water or sediment samples from both rivers, Marañón and Corrientes, clearly, posing a threat to biodiversity and human inhabitants of these oil-impacted areas.

Towns along these rivers do not have adequate infrastructure or available drinking water, and rural people have to collect their domestic water directly from the river. Neither Peru nor USEPA have standard limits for PAHs as a class in drinking water, but Europe does, which is 0.0001 µg/ml (European Communities, 2007), and this study

found that all the PAH concentrations found in water (7.54 to 210.15 µg/ml) exceed this limit.

The only PAH detected in water samples from T4 was fluoranthene. Since fluoranthene has low water solubility, and will be adsorbed to sediment and particulate matter rapidly (Williams and Taylor, 1993), its presence suggests that this contamination is derived from a local source, such as the oil activities complex by the collection sites. Site 4 in Trompeteros was located downstream of Trompeterillo stream where water containing oil is presumably disposed, and this might explain the high concentration of PAHs in the sediment sample from this site.

Several other studies in South America have determined PAH concentrations in water and sediment samples related to oil contamination. Water samples for human consumption were collected from rivers, lagoons and wells in Chaco (Bolivia), one of the most productive oil regions in the country. The total PAH concentration had a mean of 0.004 µg/ml and the values ranged from 0.0002 to 2.99 µg/ml (González Alonso et al., 2010). In Uruguay and the Plata River, the concentrations ranged from 0.0018 to 0.012 µg/ml (Barra et al., 2007). Sediment samples from Santos, a Brazilian region exposed to oil activities, showed aromatic hydrocarbons from 0.08 to 42.39 µg/ml with higher concentrations of pyrene, crysene and indeno[1,2,3-cd]pyrene indicating oil and/or incomplete combustion pollution (Nishigima et al., 2001). In Colombia, Cartagena Bay, a port where oil and fuel manipulation are continuous, Parga-Lozano et al. (2002) found hydrocarbons in sediments ranging from 100 µg/ml to 1415 µg/ml, which is approximately double ours.

Several tests have been performed in order to determine the carcinogenicity of PAHs, and the following hydrocarbons have been found to be carcinogenic or possible human carcinogens: benzo[a]anthracene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, dibenzo[a,h]anthracene, and indeno[1,2,3-cd]pyrene (IARC, 1983). In Saramuro, these chemicals contributed up to 88% of the Σ PAH concentrations for all the water samples together, 91% for sediment samples, and in Trompeteros, 82% in sediment samples.

Benzo[a]pyrene (BaP) is associated with particulate matter, soils, and sediment, with a half-life of two days to 1.9 years, and can be carried for long distances (WHO, 1999). Benzo[a]pyrene was found in all sediment samples from both San José de Saramuro and Trompeteros; while in water, it was found in four out of five samples in San José de Saramuro, and one out of six samples in Trompeteros. All the concentrations found exceeded the standard limits set by the European Communities of 0.00001 µg/ml (2007), USEPA of 0.0002 µg/ml (2011b), and El Peruano of 0.0007 µg/ml (2008). This result is worth notice since BaP has been identified as a promutagen in fish (Hawkins et al., 1990; i.e., requires metabolic activation to become a DNA-damaging agent) (Johnson, 1992). Lesions in DNA are a trigger for the carcinogenetic process, and micronuclei formation gives information about the promutagenesis of these lesions (Robbiano et al., 1999). The genotoxic potential of BaP was demonstrated by Maria et al. (2002), who found an increase of erythrocytic nuclear abnormalities (ENA) and a decrease in blood and liver DNA integrity in eel *Anguilla anguilla.* In humans, BaP has been associated with chromosomal replication (DNA copying) errors and altered DNA in gametes (sperm and eggs). It also forms BaP-DNA adducts in fetal, child, and adult tissues. At high levels

of acute exposure, BaP has been reported to be associated with immune system suppression and red blood cell damage leading to anemia (ATSDR, 1995).

Polycyclic aromatic hydrocarbons in the diesel water-soluble fraction (DSWF) are thought to form electrophilic compounds that can cause damage to DNA. Genotoxic effects of DSWF on the seahorse *Hippocampus reidi* and effects of effluents from a petroleum refinery on tilapia *Oreochomis niloticus* were assessed in Brazil, and results showed an increase of micronuclei. These errors are a result of chromosome breakage during cell division, probably induced by defects in the gene (Hoshina et al., 2008; Alcoforado Santos et al., 2010). The neotropical fish *Prochilodus lineatus* was exposed to DSWF and results indicated genotoxic and mutagenic damage in erythrocytes (Vanzella et al., 2007). Another study in Brazil found that gasoline water-soluble fraction (GWSF) damaged DNA in gill cells and hemocytes of Asian clam *Corbicula fluminea* (Fedato et al., 2010). The process of PAH biotransformation in organisms converts these pollutants into intermediary toxic reactive compounds causing DNA oxidative damage. Damage in the DNA structure could lead to mutagenicity and carcinogenicity generating alterations in present and future populations (Maria et al., 2002).

Pluspetrol Peru Corporation S.A. is not the only oil company polluting the Corrientes River. An important and constant source of contamination is the upstream Ecuadorian oil industry since the river originates in this neighboring country (Laraque et al., 2007). Crude oil extraction began in Ecuador more than 40 years ago and has become a major source of income for the country, as well as a source of environmental and health problems (San Sebastián and Hurtig, 2004). For many years concerns have

been raised reporting declines in edible fish populations in local streams and rivers, cattle dying from contaminated water, and skin rashes in people after bathing in the river (Kimberling, 1995). In the Rumiyacu River in Ecuador, total petroleum hydrocarbon (TPH) concentration in sediments ranged from 4.9 to 6980 µg/ml, and water samples ranged from 0.05 to 0.12 µg/ml, above the permitted limit for hydrocarbons in drinking water in Ecuador (0.01 µg/ml; Wernersson, 2004). Another study found TPH concentrations in San Carlos, a small village with more than 30 oil wells surrounding it in northeastern Ecuador, to be between 0.09 and 2.88 µg/ml, suggesting that this severe water contamination is linked with the higher-than-expected cancer mortality in the village (San Sebastián et al., 2001a). Studies on the Ecuadorian Amazon have reported skin mycosis, ear pain, gastritis (San Sebastián et al. 2001b), increase of spontaneous abortions (San Sebastián et al., 2002), child leukemia (Hurtig and San Sebastián, 2004), and elevated stomach, rectum, kidney and cervix cancer (Hurtig and San Sebastián, 2002), all related to living in vicinity to oil activities.

MUTA-CHROMOPLATE™

WATER AND SEDIMENT SAMPLES. The positive mutagenic responses of this study suggests that the water in San José de Saramuro, and water and sediment in Villa Trompeteros contain mutagens that may pose risks of unknown magnitude to organisms and people along the river. A higher mutation ratio in TA98 suggests that the water samples contain mostly frameshift mutagens, even compared to the mutation ratio in sediment samples. Frameshift mutation or framing error is the insertion or deletion of a base or bases into the genome causing a change in the reading frame (Streisinger et al., 1966). Other studies have shown that samples related to oil and aromatic hydrocarbons

have higher mutation ratios using TA98. In Alaska, Prudhoe Bay crude oil was tested, and was found to be mutagenic using strain TA98 (Sheppard et al., 1983). Individual PAHs have been tested and it was found that benzo[a]pyrene, benzo[g,h,i]perylene, fluoranthene, indeno[1,2,3,-cd]pyrene, and pyrene are mutagenic to *Salmonella* using strain TA98 (Waldron and White, 1989). Water samples in Saramuro that were found mutagenic contained at least one of these PAHs. Despite the fact that no hydrocarbons were detected in the water samples from Trompeteros (T2 and T3) tested for mutagenicity, they were positive, indicating that there may be other mutagenic pollutants in these sites. In sediment, site 4 (Trompeteros) was the only sample that tested positive, probably because it had the highest Σ PAH concentration.

Hydrocarbons could have different activation mechanisms or not have any at all. In Slovenia, a study of industrial and domestic wastewater found that extracts contained nitropolyaromatic hydrocarbons and were mutagenic to strain TA98 without metabolic activation (S9) (Filipic and Toman, 1996). The water and sediment samples collected in the present study were not tested with S9 either, and three of the four tested sediment samples in Trompeteros were not mutagenic. These results could be treated as "false negatives", where some mutagens require metabolic activation to be detected or they elicit their effects through a non-mutagenic mechanism (Greim et al., 1980). This metabolic activation results from interaction with microsomal enzymes present in many cells, producing epoxides that react with DNA and produce mutations in the count frame shift (Pashin and Bakhitova, 1979). In another study, Yan et al. (2004) exposed *Salmonella typhimurium* using strain TA102 to PAHs and light (1.1 J/cm^2 UVA + 2.1 J/cm^2 visible), and anthracene, benz[a]anthracene, benzo[ghi]perylene, benzo[a]pyrene,

indeno[1,2,3-cd]pyrene, and pyrene were found photomutagenic. The same PAHs were tested using S9 and were not mutagenic.

CRUDE OIL. Lockard et al. (1982) tested different oils using the *Salmonella*/microsome mutagenicity assay, and suggested that the Wilmington crude oil (from Delaware, U.S.A.) was not mutagenic, Eastern U.S. shale oil (kerogen oil produced by pyrolysis or hydrogenation) had weak mutagenicity, and coal-derived oil was mutagenic, more active with TA98 and S9 activation. An *in vitro* sister chromatid exchange (SCE) assay, which detects exchanges of DNA between two sister chromatids, was also performed and the number of mutational events were consistent with the mutagenicity levels of each oil. Coal-derived oil produced from liquefaction has a higher carcinogenic and mutagenic level than crude petroleum, and the basic and neutral fractions have the most mutagenic activity (Kimball and Munro, 1981). In the present study, two strains were used for assessing Peruvian crude oil, TA98 and TA100, with and without metabolic activation of the test compound (S9), and the sample was found mutagenic using TA98 with S9 activation. Mutagens requiring metabolic activation by microsomal (S9) enzymes to become genetically active are called promutagens. Many natural products (plant allelochemicals and mycotoxins), aliphatic vinyl-compounds, aromatic amines and PAHs undergo metabolic activation to reactive electrophiles (Venitt and Parry, 1984).

The mutagenicity of water and sediment samples and the low mutagenicity of the water accommodated fraction (WAF) using Peruvian crude oil suggest that the sampled areas contain additional contaminants affecting the rivers. Vandermeulen et al. (1985) tested different oils such as Saran Gach from Iran and Kuwait crude, diesel 25, and

Bunker C, a residual fuel. The water-soluble fractions (WSF) of oil products showed low mutagenicity, suggesting that the toxicity of some components might be masking the mutagenic activity of others. This contradiction in results could also be due to the potential problem that crude oil is a complex chemical mixture and sensitivity may be lost, since mutagenicity of the whole material could be less than individual components (Pelroy and Petersen, 1979). For instance, Pelroy and Petersen (1979) separated crude shale oil into five compounds and results showed that basic and PAH fractions were more mutagenic than the crude shale oil by itself. Manabe et al. (1984) tested oil contaminated water, fractioned it into neutral, acidic and basic components, and found that the neutral fractions showed the highest mutagenicity using strains TA98 and TA100.

MICROTOX®

WATER AND SEDIMENT SAMPLES. It is necessary to prescreen oils and contaminants that could pose a danger to organisms. The Microtox® assay and other similar tests such as, the standard *Hyalella azteca* bioassay, the Biotox™ Flash test, the Ostracodtoxkit assay, and algal assay have been useful in detecting toxicity of oil-contaminated sediments (Blaise et al., 2004). Microtox® has also been used in testing toxicity before and after bioremediation associated with oil contamination (Dorn and Salanitro, 2000; Delille at al., 2002). It also shows a consistent increasing response with increasing oil levels (van Gestel et al., 2001). In the present study, one water sample (T4) was found acutely toxic, which was located on a stream where oil contaminated water and untreated wastewater is discharged. This result is also consistent with the fluoranthene concentration found in the sample; it was the highest of all, and aqueous solutions of aromatic hydrocarbons larger than fluoranthene (3-ring) have low aqueous

solubilities that may not be acutely toxic (Di Toro et al., 2007). In addition, three sediment samples were found acutely toxic, compared to one water sample. The sediment sample from T4 had the lowest EC_{50} of all the samples, and the highest Σ PAH concentration in the Trompeteros area samples. Sediments are a sink for organic chemicals and PAHs tend to accumulate in sediment, affecting the aquatic environment (Tollefsen et al., 2006). Delille et al. (2002) analyzed Arabian Light crude contaminated interstitial water and no toxicity was found. However, high toxicity was found in contaminated sediments analyzed even after one year of bioremediation treatment suggesting that oil pollutants stay in sediments after a long period of time. The EC_{50} values for sediment in the present study ranged from 25.67 to 335.1 mg/L. According to Doe et al. (2005), sediments with EC_{50} values $\leq$ 1000 mg/L are toxic.

CRUDE OIL. The toxicity of the water accommodated fraction (WAF) using Peruvian crude oil (EC_{50} = 17.18 mg/L) was lower than the water and sediment samples tested, possibly because the sample of crude oil was more concentrated (200 g oil/L water) and sediment samples contained other potential contaminants. In Brazil, the soluble crude oil fraction was tested and also showed acute toxicity. In addition, it was suggested that photocatalysis is a potential process for water treatment eliminating crude oil compounds toxicity (Ziolli and Jardim, 2002). Depending on the type of crude oil, toxicity varies. Hokstad et al. (1999) tested WAF (25 g oil/L seawater) from Statfjord and Troll crude oil, and found the 5-minute EC_{50} to be 2.08 mg/L and 1.08 mg/L, respectively. Faksness et al. (2012) found a similar EC_{50} value for Troll crude oil, 1.1 mg/L. Arabian Light crude WAF was tested using Microtox® for 15-minute exposures and the EC_{50} was 1.0 mg/L, thus, less toxic than the North Sea's crude oils (Fuller and

Bonner, 2001). All these studies found EC_{50} values lower than the value found for Peruvian crude oil, therefore, were more toxic.

CONCLUSIONS

This study reported the measurement of PAH concentrations, EC_{50} obtained from Microtox® Acute Toxicity Test, and mutagenicity of oil-contaminated water and sediment from two areas in the Peruvian Amazon, and Peruvian crude oil. The highest total PAH concentration near Saramuro was found in water, 210.15 µg/ml, and the highest in Trompeteros was also found in water, 204.66 µg/ml. All water samples tested for Saramuro and Trompeteros, and one sediment sample were found to be mutagenic for both strains TA98 and TA100. Peruvian crude oil was mutagenic using strain TA98 and S9 enzyme, suggesting that it contains chemicals that require enzymatic activation. However, the sample did not show significant mutagenicity without S9. Results from the Microtox Test showed that there was a sediment sampled that was toxic in Saramuro (EC_{50} = 335.1 mg/l), and in Trompeteros toxicity ranged from 25.67 to 133.86 mg/l, one in water and two in sediment samples. The EC_{50} for WAF using Peruvian crude oil was 17.18 mg/l. The two areas sampled had very high PAH concentrations that are most likely associated with oil activities. The results in the present study suggested that even though the water and sediment samples collected contained PAHs, the toxic effects were not acute. However, since most of the samples were mutagenic, it is thought that the DNA structure could be damaged. This confirms that wastewater containing oil is comprised of harmful substances; including those with genotoxic and carcinogenic effects, and that the oil industry in Peru has the potential and may be severely degrading aquatic organisms and people's health. These are alarming results that should be considered since there are organisms and indigenous people that depend on these rivers and its tributaries.

Additional assays are recommended in order to determine all the contaminants present in the water and sediment of these rivers, such as heavy metals. Thus, concentration of the 16 priority PAHs in fish samples from different sites in both rivers should be determined. Microbial, chemical, and more toxicological tests are necessary to predict the effects of oil and implement bioremediation methods to alleviate the damage that the oil industry has caused in this part of Peru.

Table 3.1. Collection sites on the Marañón River near San José de Saramuro in Loreto, Peru, sampled during summer 2011.

Site number	S1	S2	S3	S4	S5
Date	6/13/2011	6/13/2011	6/14/2011	6/14/2011	6/13/2011
Time	16:04	13:21	8:20	9:01	14:18
GPS coordinates	S 4º 42' 37.0" W 074º 56' 33.2"	S 4º 43' 06.4" W 074º 55' 33.6"	S 4º 43' 37.7" W 074º 55' 08.5"	S 4º 44' 28.3" W 074º 54' 34.1"	S 4º 53' 57.2" W 074º 54' 41.7"
Weather conditions	Sunny, partially cloudy	Sunny, partially cloudy, scattered showers	Cloudy, rained overnight	Cloudy, rained overnight	Scattered showers, sunny, partially cloudy
Site description	Upstream 3 km from main pipeline. Side of branch, just outside weeds, out of current about 1 – 2 m from shoreline, and 1 – 3 m depth.	Upstream 1 km from main pipeline. Island, main branch next to vegetation, out of current about 1 – 2 m from shoreline, and 1 – 3 m depth.	Downstream 100 m from main pipeline. Island, main branch next to vegetation, out of current about 1 – 2 m from shoreline, and 1 – 3 m depth. Water level increased due to rain overnight.	Downstream 150 m from 2nd pipeline. Pacaya-Samiria side, main branch close to vegetation, out of current about 1 – 2 m from shoreline, and 1 – 3 m depth.	Downstream 1 km from 2nd pipeline. Island, main branch next to vegetation, out of current about 1 – 2 m from shoreline, and 1 – 3 m depth.

Table 3.2. Collection sites on the Corrientes River near Villa Trompeteros in Loreto, Peru, sampled during summer 2011.

Site number	T1	T2	T3	T4	T5	T6
Date	6/26/2011	6/26/2011	6/26/2011	6/26/2011	6/26/2011	6/26/2011
Time	6:45	12:25	12:25	7:35	8:35	9:50
GPS coordinates	S 3º 48' 44.4" W 075º 04' 29.9"	S 3º 48' 51.6" W 075º 04' 05.7"	S 3º 48' 51.6" W 075º 04' 05.7"	S 3º 48' 24.6" W 075º 03' 27.6"	S 3º 48' 26.9" W 075º 01' 47.9"	S 3º 48' 26.3" W 075º 01' 31.6"
Weather condition	Cloudy	Cloudy	Cloudy	Cloudy	Cloudy	Cloudy
Site description	Upstream 1 km from Trompeterillo stream. Island, main branch next to vegetation, out of current, about 1 – 2 m from shoreline, and 1 – 3 m depth.	Inside Trompeterillo stream. 30 m from Corrientes River. Security staff from oil facility did not allow sediment sampling.	Just outside Trompeterillo stream, 1 m from yellow flotation limit. Security staff from oil facility did not allow sediment sampling.	Downstream 1.5 km from Trompeterillo stream. Shore was too steep. Dredge could not be used. A scoop was used on shoreline.	Trompeteros stream, 100 m from the Corrientes River. Inside stream, sampling in the middle about 2 m from shoreline, and 1–3 m depth.	Downstream 500 m from Trompeteros stream. Shore was too steep. Dredge could not be used. A scoop was used on shoreline.

Table 3.3. Assay preparation for controls and duplicate study samples (Saramuro = San José de Saramuro, Trompeteros = Villa Trompeteros) in *Salmonella* strains TA98 and TA100. Note: 2-NF = 2-nitrofluorine and NaN_3 = sodium azide.

	Standard (ml)	Sample (ml)	H_2O (ml)	Reaction mix (ml)	Bacteria (5 µl)
Blank	0.0	0.0	17.5	2.5	None
Background 1	0.0	0.0	17.5	2.5	TA98
Background 2	0.0	0.0	17.5	2.5	TA100
2 - NF	0.1	0.0	17.4	2.5	TA98
NaN_3	0.1	0.0	17.4	2.5	TA100
Water Samples					
Saramuro 2	0.0	15.0	2.5	2.5	TA98
Saramuro 2	0.0	15.0	2.5	2.5	TA100
Saramuro 3	0.0	15.0	2.5	2.5	TA98
Saramuro 3	0.0	15.0	2.5	2.5	TA100
Saramuro 5	0.0	15.0	2.5	2.5	TA98
Saramuro 5	0.0	15.0	2.5	2.5	TA100
Trompeteros 2	0.0	15.0	2.5	2.5	TA98
Trompeteros 2	0.0	15.0	2.5	2.5	TA100
Trompeteros 3	0.0	15.0	2.5	2.5	TA98
Trompeteros 3	0.0	15.0	2.5	2.5	TA100
Sediment Samples					
Trompeteros 1	0.0	15.0	2.5	2.5	TA98
Trompeteros 1	0.0	15.0	2.5	2.5	TA100
Trompeteros 4	0.0	15.0	2.5	2.5	TA98
Trompeteros 4	0.0	15.0	2.5	2.5	TA100
Trompeteros 5	0.0	15.0	2.5	2.5	TA98
Trompeteros 5	0.0	15.0	2.5	2.5	TA100
Trompeteros 6	0.0	15.0	2.5	2.5	TA98
Trompeteros 6	0.0	15.0	2.5	2.5	TA100

Table 3.4. Assay preparation for controls and sample duplicates (water accommodated fraction with 200 g/l Peruvian crude oil) in *Salmonella* strains TA98 and TA100 with and without metabolic activation (S9 enzyme). Note: 2-NF = 2-nitrofluorine, NaN_3 = sodium azide and 2-AA = 2-amino anthracene.

	Standard (ml)	Sample (ml)	H_2O (ml)	Reaction mix (ml)	S9 (ml)	Bacteria (5 µl)
Blank	0.0	0.0	17.5	2.5	None	None
Background 1	0.0	0.0	17.5	2.5	None	TA98
Background 2	0.0	0.0	15.5	2.5	2.0	TA98
Background 3	0.0	0.0	17.5	2.5	None	TA100
Background 4	0.0	0.0	15.5	2.5	2.0	TA100
2 - NF	0.1	0.0	17.4	2.5	None	TA98
NaN_3	0.1	0.0	17.4	2.5	None	TA100
2- AA	0.1	0.0	15.4	2.5	2.0	TA100
Crude oil	0.0	15.0	2.5	2.5	None	TA98
Crude oil	0.0	15.0	0.5	2.5	2.0	TA98
Crude oil	0.0	15.0	2.5	2.5	None	TA100
Crude oil	0.0	15.0	0.5	2.5	2.0	TA100

Table 3.5. Sixteen Polycyclic Aromatic Hydrocarbons and Σ PAH concentrations in water and sediment samples from five collection sites on the Marañón River near San José de Saramuro in Loreto, Peru, sampled during summer 2011. Note: Result (µg/ml) is the average of three replicates ± standard deviation, nd = not detected (detection limit: 1 µg/l), Σ = sum.

	WATER					SEDIMENT				
Polycyclic Aromatic Hydrocarbons	S1	S2	S3	S4	S5	S1	S2	S3	S4	S5
Naphthalene	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Acenaphthylene	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Acenaphthene	nd	nd	0.46 ± 0.79	nd	nd	nd	nd	nd	nd	nd
Fluorene	nd	nd	33.11 ± 31.27	nd	nd	nd	nd	nd	nd	nd
Phenanthrene	nd	nd	3.82 ± 1.70	nd	nd	nd	nd	nd	nd	22.14 ± 38.35
Anthracene	nd	nd	0.37 ± 0.64	3.13 ± 5.42	nd	nd	nd	nd	nd	0.84 ± 1.45
Fluoranthene	nd	nd	nd	nd	nd	nd	nd	4.32 ± 3.96	1.12 ± 1.93	nd
Pyrene	nd	nd	3.47 ± 0.74	4.03 ± 2.08	nd	nd	nd	3.86 ± 3.96	nd	nd
Benz[a]anthracene	nd	nd	1.19 ± 2.06	nd	nd	nd	nd	nd	nd	11.80 ± 20.44
Chrysene	nd	nd	2.00 ± 0.99	12.48 ± 6.44	nd	nd	nd	0.55 ± 0.96	nd	0.39 ± 0.68
Benzo[b]fluoranthene	nd	nd	18.61 ± 10.29	13.87 ± 12.21	1.41 ± 2.44	nd	nd	0.44 ± 0.76	nd	nd
Benzo[k]fluoranthene	nd	nd	21.62 ± 13.95	2.56 ± 2.98	nd	nd	nd	7.77 ± 0.75	nd	23.33 ± 21.31
Benzo[a]pyrene	nd	1.23 ± 1.16	41.59 ± 18.10	21.07 ± 9.99	6.14 ± 2.09	2.56 ± 2.44	2.19 ± 1.91	16.42 ± 1.40	12.03 ± 5.05	20.95 ± 10.89
Dibenzo[a,h]anthracene	nd	8.67 ± 15.02	95.69 ± 31.16	38.86 ± 8.36	nd	nd	nd	nd	nd	13.09 ± 8.22
Indeno[1,2,3-cd]pyrene	nd	nd	22.37 ± 13.95	4.43 ± 1.57	nd	nd	nd	nd	nd	nd
Benzo[ghi]perylene	nd	nd	3.25 ± 1.57	6.38 ± 2.68	nd	nd	nd	nd	nd	nd
Σ PAH concentration	nd	9.90	210.15	104.81	7.54	2.56	2.19	33.36	13.14	70.41

Table 3.6. Sixteen Polycyclic Aromatic Hydrocarbons and Σ PAH concentrations in water and sediment samples from six collection sites on the Corrientes River near Villa Trompeteros in Loreto, Peru, sampled during summer 2011. Note: Result (µg/ml) is the average of three replicates ± standard deviation, nd = not detected (detection limit: 1 µg/l), Σ = sum.

	WATER						SEDIMENT			
Polycyclic Aromatic Hydrocarbons	T1	T2	T3	T4	T5	T6	T1	T4	T5	T6
Naphthalene	nd	nd	nd	nd	nd	2.7 ± 2.21	nd	nd	nd	nd
Acenaphthylene	nd	nd	nd	nd	nd	5.69 ± 1.65	nd	nd	nd	nd
Acenaphthene	nd	nd	nd	nd	nd	5.76 ± 4.22	nd	nd	nd	nd
Fluorene	nd	nd	nd	nd	nd	10.60 ± 4.42	nd	nd	nd	nd
Phenanthrene	nd	nd	nd	nd	nd	8.30 ± 13.58	nd	nd	nd	nd
Anthracene	nd	nd	nd	nd	nd	70.08 ± 56.46	nd	nd	nd	nd
Fluoranthene	nd	nd	nd	20.71 ± 1.80	nd	27.62 ± 22.57	nd	10.62 ± 3.62	2.36 ± 2.18	3.82 ± 3.35
Pyrene	nd	nd	nd	nd	nd	2.90 ± 1.14	nd	0.68 ± 1.18	nd	0.48 ± 0.83
Benz[a]anthracene	nd	nd	nd	nd	nd	0.21 ± 0.23	nd	nd	nd	nd
Chrysene	nd	nd	nd	nd	nd	5.84 ± 6.65	nd	nd	nd	nd
Benzo[b]fluoranthene	nd	nd	nd	nd	nd	2.58 ± 1.33	nd	nd	nd	nd
Benzo[k]fluoranthene	nd	nd	nd	nd	nd	2.56 ± 2.55	nd	nd	0.90 ± 1.55	0.87 ± 1.50
Benzo[a]pyrene	nd	nd	nd	nd	nd	0.93 ± 0.43	3.59 ± 6.22	44.90 ± 9.38	8.36 ± 1.50	9.73 ± 4.24
Dibenzo[a,h]anthracene	nd	nd	nd	nd	nd	47.67 ± 33.88	nd	11.13 ± 14.63	nd	nd
Indeno[1,2,3-cd]pyrene	nd	nd	nd	nd	nd	7.48 ± 4.85	nd	nd	nd	nd
Benzo[ghi]perylene	nd	nd	nd	nd	nd	3.76 ± 4.53	nd	nd	nd	nd
Σ PAH concentration	nd	nd	nd	20.71	nd	204.66	3.59	67.33	11.62	14.90

Table 3.7. Mutagenic profiles and median effective concentrations (EC_{50}) of water and sediment samples collected from San José de Saramuro and Villa Trompeteros using the *Salmonella* fluctuation test. Note: NTACT = No toxicity at concentration tested, SD = standard deviation (if 0.00: all 96-well plate was converted), MR = Mutation Ratio, NS = Not significant, (-) = not done.

	EC_{50} (mg/l)	Bacteria strain (*Salmonella*)	Test plate positives (SD)	Negative control plate positives	MR	Significance
Water samples						
Saramuro 1	NTACT	-	-	-	-	-
Saramuro 2	NTACT	TA98	96 (0.00)	6	16	<0.001
		TA100	96 (0.00)	10	9.6	<0.001
Saramuro 3	NTACT	TA98	94.5 (0.71)	6	15.75	<0.001
		TA100	94 (0.00)	10	9.4	<0.001
Saramuro 4	NTACT	-	-	-	-	-
Saramuro 5	NTACT	TA98	95.5 (0.71)	6	15.92	<0.001
		TA100	96 (0.00)	10	9.6	<0.001
Trompeteros 1	NTACT	-	-	-	-	-
Trompeteros 2	NTACT	TA98	96 (0.00)	6	16	<0.001
		TA100	96 (0.00)	10	9.6	<0.001
Trompeteros 3	NTACT	TA98	96 (0.00)	6	16	<0.001
		TA100	96 (0.00)	10	9.6	<0.001
Trompeteros 4	133.86	-	-	-	-	-
Trompeteros 5	NTACT	-	-	-	-	-
Trompeteros 6	NTACT	-	-	-	-	-
Sediment samples						
Saramuro 1	NTACT	-	-	-	-	-
Saramuro 2	335.1	-	-	-	-	-
Saramuro 3	NTACT	-	-	-	-	-
Saramuro 4	NTACT	-	-	-	-	-
Saramuro 5	NTACT	-	-	-	-	-
Trompeteros 1	NTACT	TA98	3 (1.41)	6	0.5	NS
		TA100	11.5 (3.54)	10	1.15	NS
Trompeteros 4	25.67	TA98	76 (1.41)	6	12.67	<0.001
		TA100	67 (1.41)	10	6.7	<0.001
Trompeteros 5	69.38	TA98	3.5 (0.71)	6	0.58	NS
		TA100	9.5 (3.54)	10	0.95	NS
Trompeteros 6	NTACT	TA98	3 (1.41)	6	0.5	NS
		TA100	10.5 (7.78)	10	1.05	NS

Table 3.8. Mutagenic profile and median effective concentration (EC_{50}) of water accommodated fraction (WAF) with 200 g/l Peruvian crude oil using the *Salmonella* fluctuation test, strains TA98 and TA100 with and without metabolic activation (S9 enzyme). Note: EC_{50} value is the average of three replicates, SD = standard deviation, MR = Mutation Ratio, NS = Not significant, (-) = not done.

	EC_{50} (mg/l)	S9	Bacteria strain *Salmonella*	Test plate positives (SD)	Negative control plate positives	MR	Significance
Crude oil	17.18	None	TA98	6 (2.12)	20	0.30	NS
		Yes	TA98	95 (0.71)	65	1.46	<0.001
		None	TA100	3 (1.41)	12	0.25	NS
		Yes	TA100	11 (1.41)	86	0.13	NS

Table 3.9. Polycyclic aromatic hydrocarbons concentration (μg/ml) in water and sediment from different locations in South America.

Sample	Location	PAH concentration (μg/ml)	Reference
Water	Uruguay and Plata River	0.0018 – 0.012	Barra et al., 2007
	Patagonia coastline	0.008 – 0.041	Barra et al., 2007
	Chaco - Bolivia	0.002 –- 2.99	González Alonso et al., 2010
	Corrientes River upstream	0.222	Goldman et al., 2007
	San José de Saramuro	nd – 210.15	Present study
	Villa Trompeteros	nd – 204.66	Present study
Sediment	Santos - Brazil	0.08 – 42.39	Nishigima et al., 2001
	Cartagena Bay - Colombia	100.00 – 1415.00	Parga-Lozano et al., 2002
	San José de Saramuro	2.56 – 70.41	Present study
	Villa Trompeteros	3.59 – 67.33	Present study

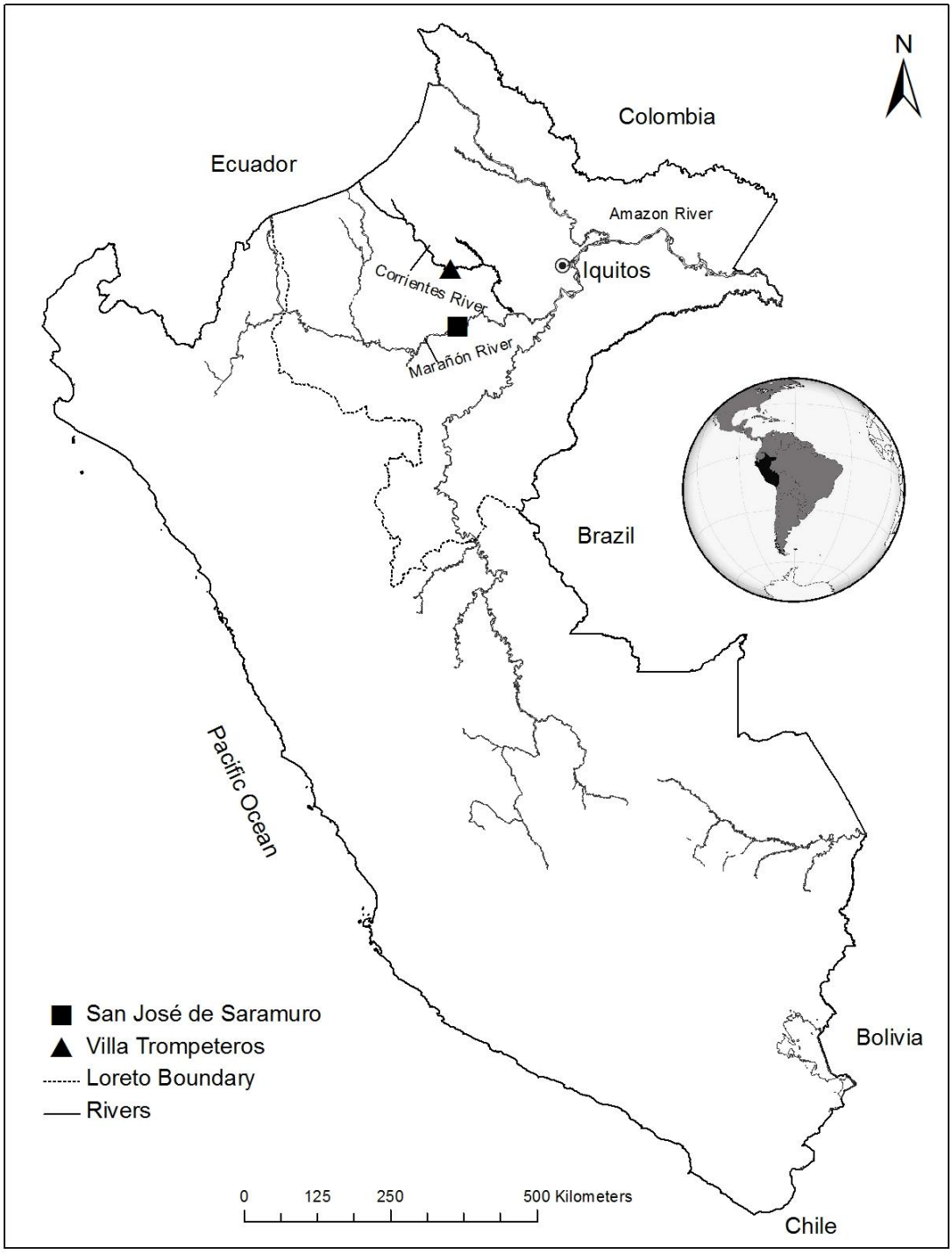

Figure 3.1 Map of Peru showing the location of the collecting sites on the Marañón River and the Corrientes River in Loreto, Peru sampled during summer 2011.

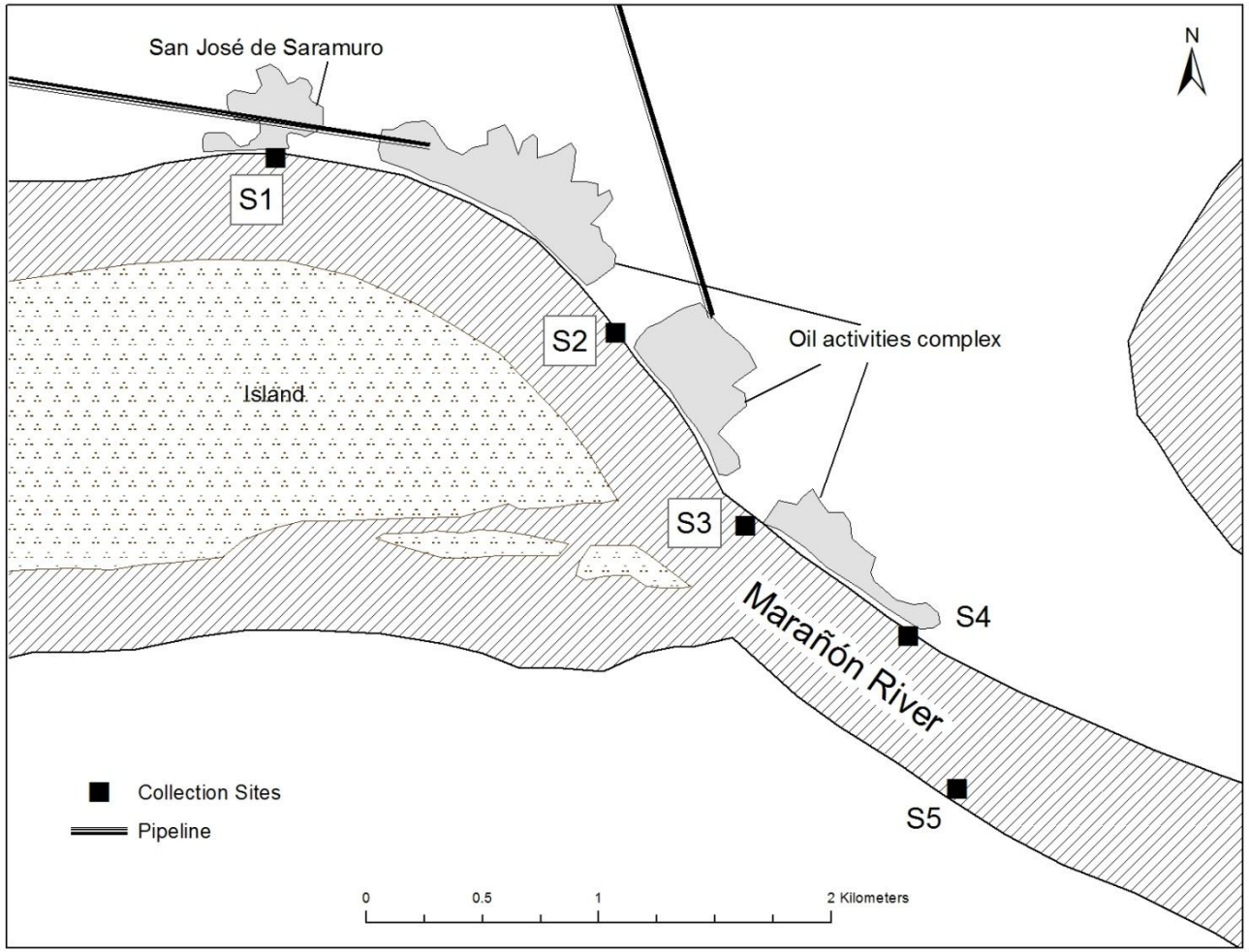

Figure 3.2 Map of San José de Saramuro and five collection sites on the Marañón River in Loreto, Peru, sampled during summer 2011.

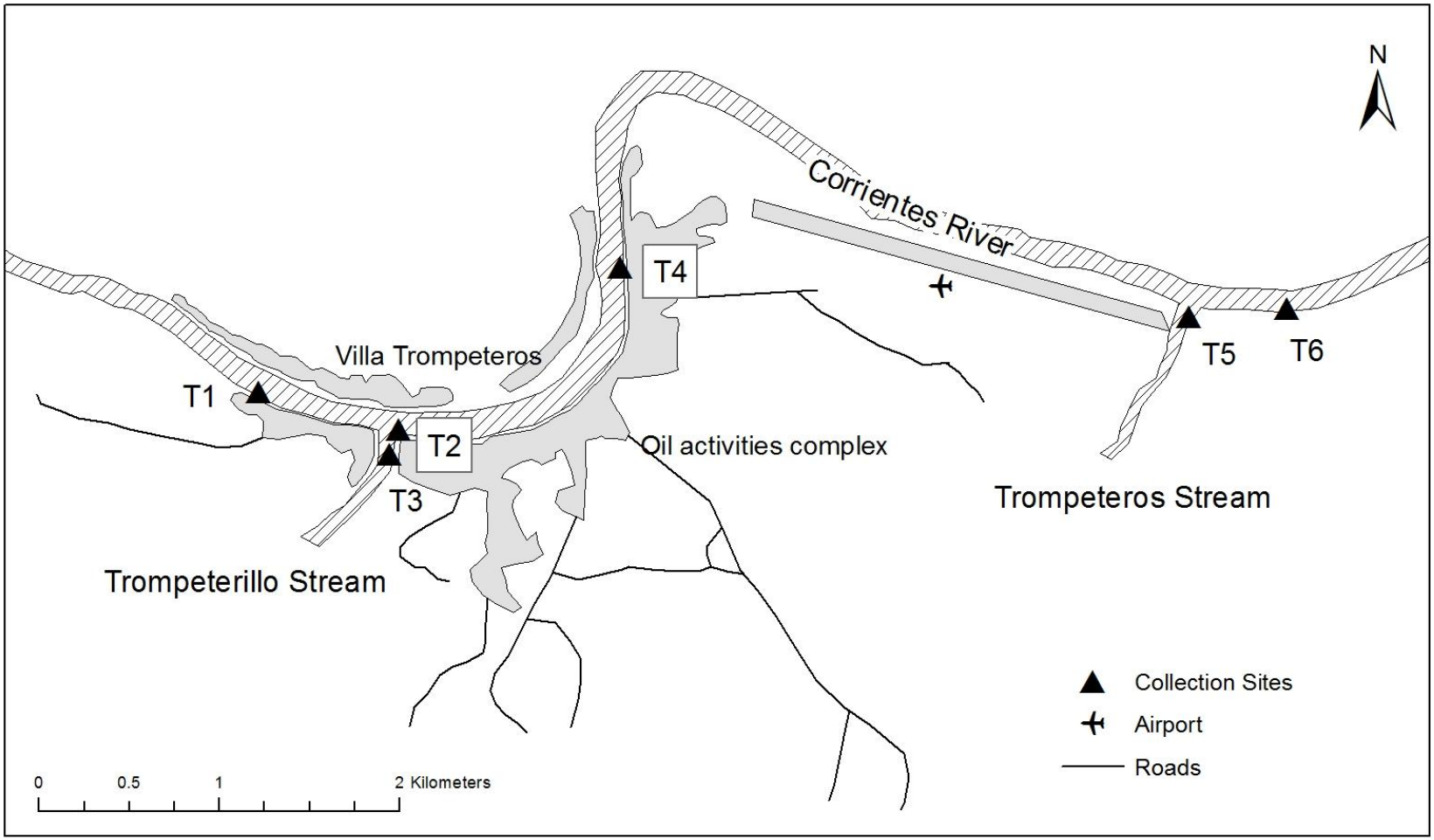

Figure 3.3. Map of Villa Trompeteros and six collection sites, on the Corrientes River in Loreto, Peru, sampled during summer 2011.

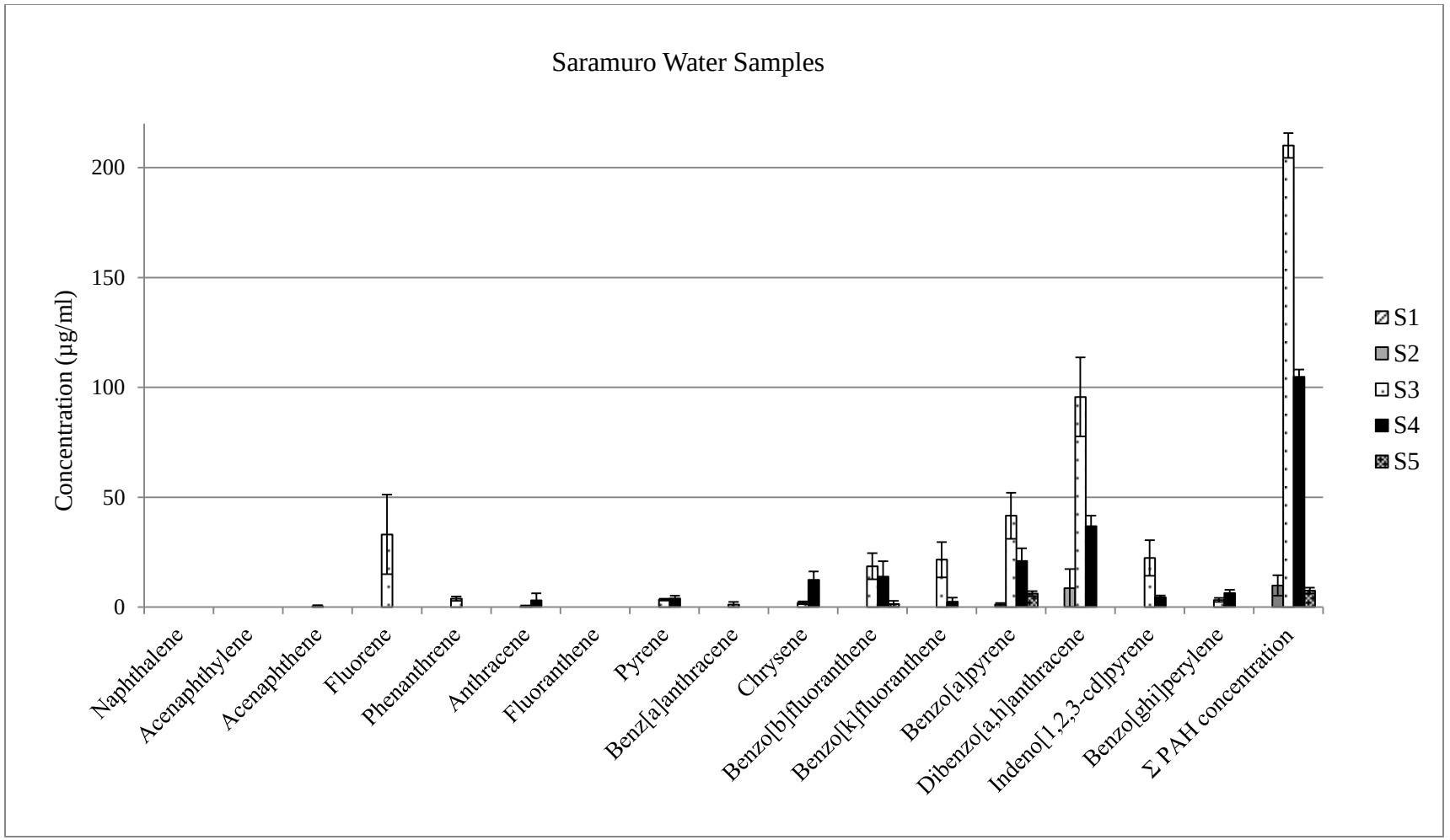

Figure 3.4 Sixteen priority Polycyclic Aromatic Hydrocarbons (PAHs) and the sum (Σ) of PAH concentrations in water samples from five collection sites (S1 – S5) on the Marañón River near San José de Saramuro in Loreto, Peru, sampled during summer 2011.

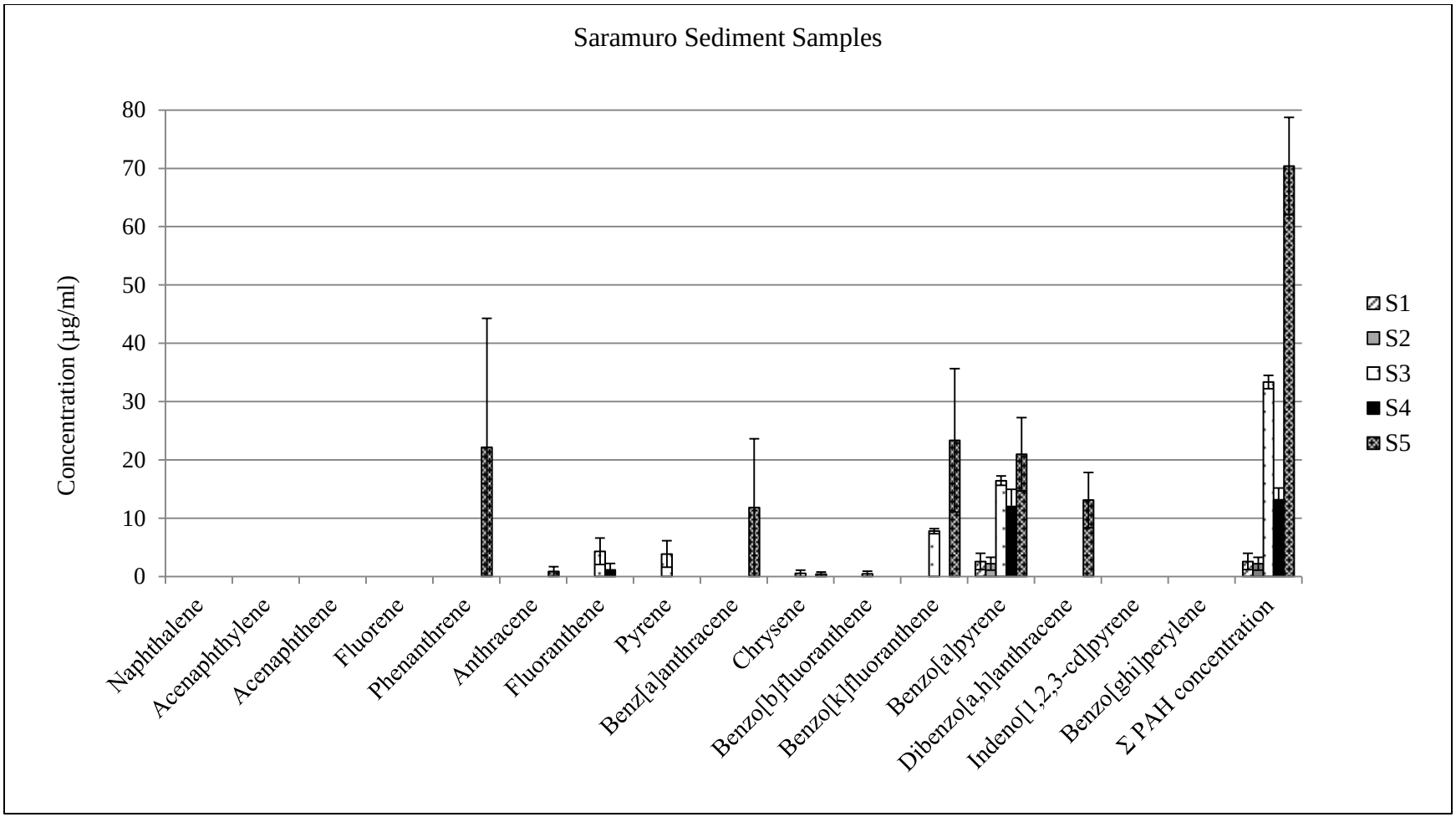

Figure 3.5. Sixteen priority Polycyclic Aromatic Hydrocarbons (PAHs) and the sum (Σ) of PAH concentrations in sediment samples from five collection sites (S1 – S5) on the Marañón River near San José de Saramuro in Loreto, Peru, sampled during summer 2011.

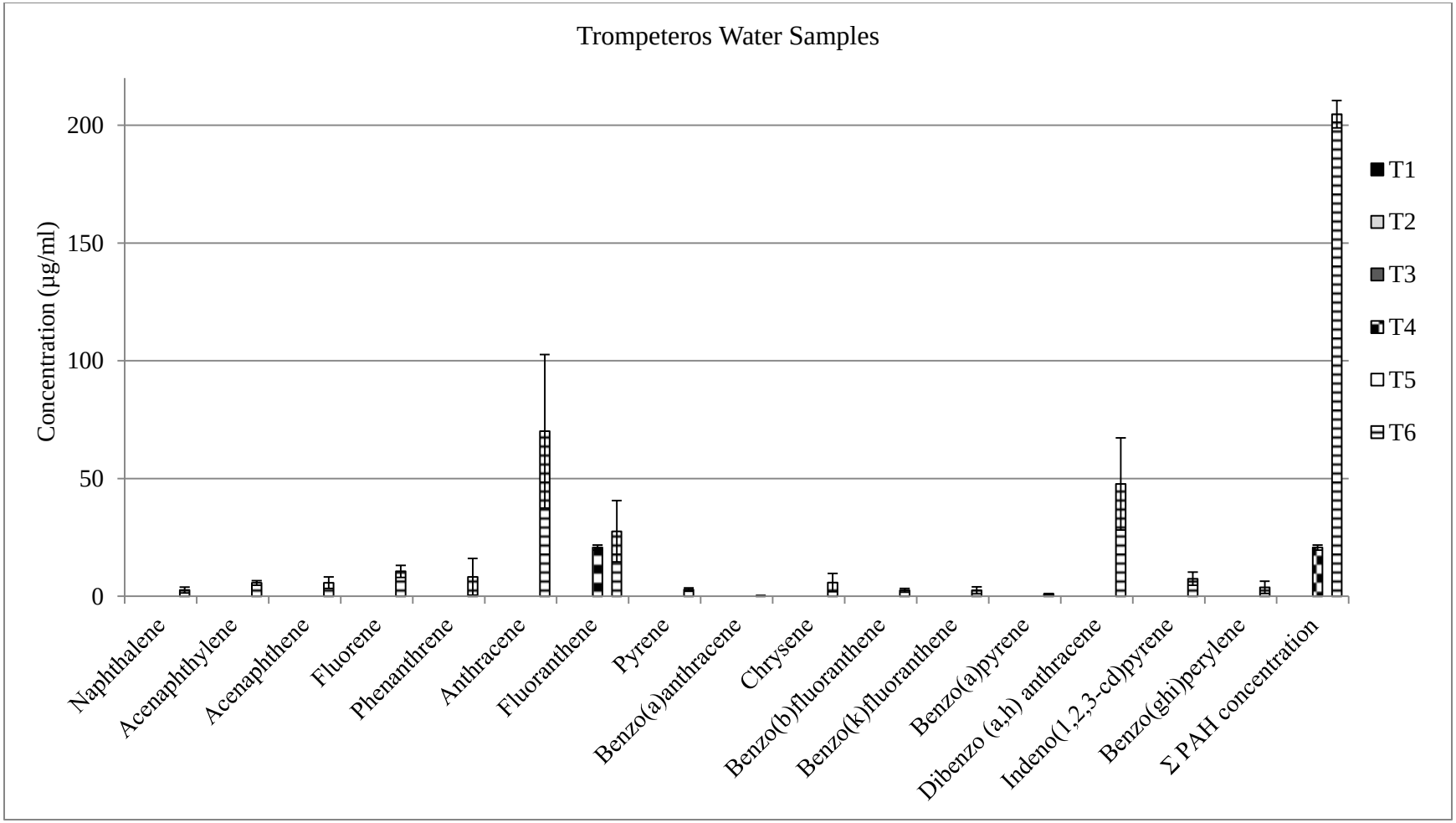

Figure 3.6. Sixteen priority polycyclic aromatic hydrocarbons (PAHs) and the sum (Σ) of PAH concentrations in water samples from six collection sites (T1 – T6) on the Corrientes River near Villa Trompeteros in Loreto, Peru, sampled during summer 2011.

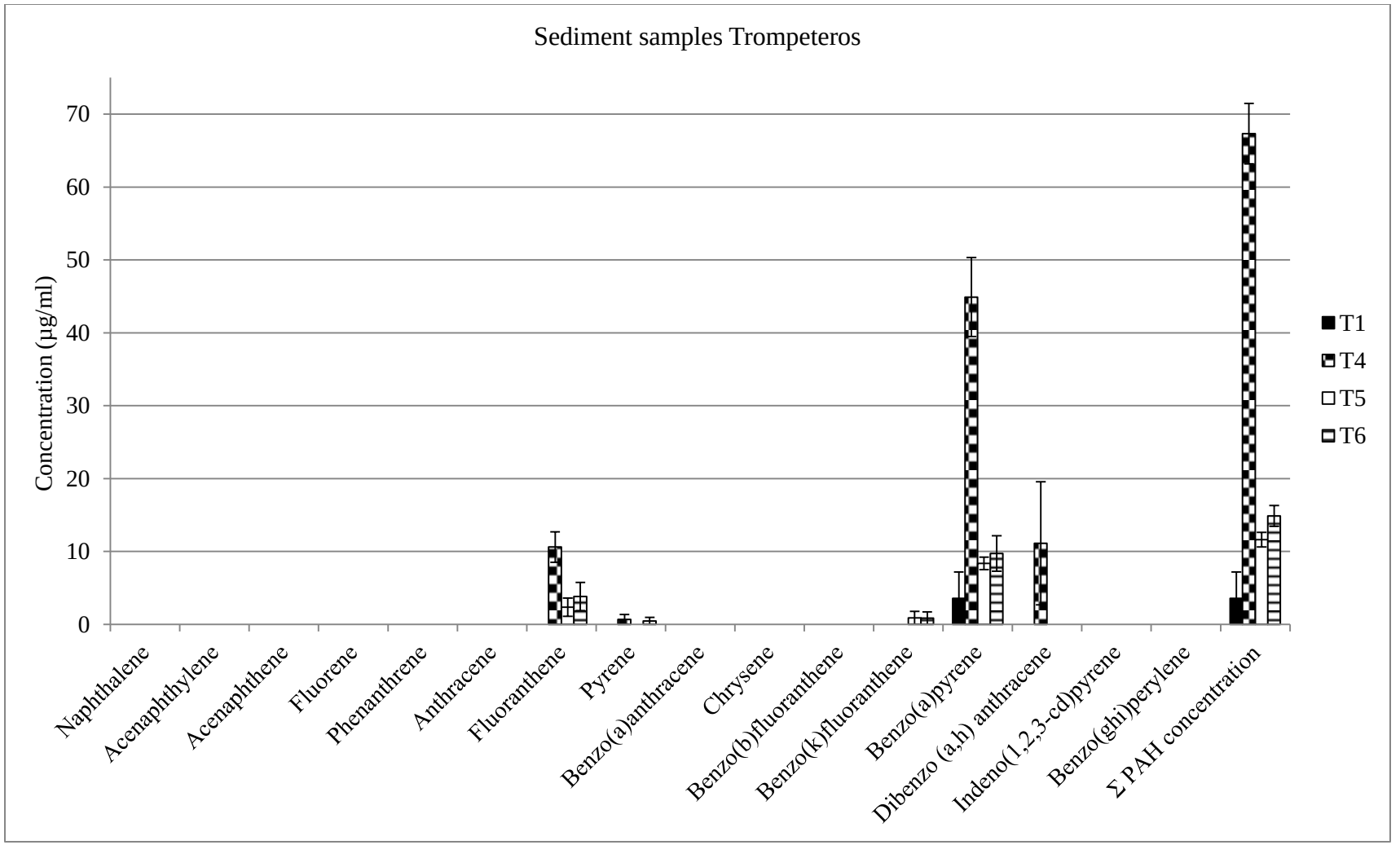

Figure 3.7 Sixteen priority Polycyclic Aromatic Hydrocarbons (PAHs) and the sum (Σ) of PAH concentrations in sediment samples from four collection sites (T1, T4 – T6) on the Corrientes River near Villa Trompeteros in Loreto, Peru, sampled during summer 2011.

CHAPTER 4 – SUMMARY AND CONCLUSIONS

This study reported LC_{50} values (Chapter 2) on a native fish species, red pacu *Piaractus brachypomus*, for three reference toxicants, zinc sulfate = 5.74 mg/l, sodium dodecyl sulfate = 11.29 mg/l, and Louisiana sweet crude oil = 2.05 mg TPH/l. When testing crude oil, it is recommendable to report the LL_{50} to better compare the results to other studies. Peruvian crude oil was tested on *Piaractus brachypomus*, and the LC_{50} was found to be > 4.00 mg TPH/l, and the LL_{50} was found to be > 50000 mg/l. The same Peruvian crude oil was tested on fathead minnows *Pimephales promelas* and the LC_{50} was 1.83 mg TPH/l, while the LL_{50} was found to be 22920 mg/l.

Piaractus brachypomus was found to be more tolerant to the Peruvian crude oil than *Pimephales promelas*. Regarding the two crude oils used, it was found that the Louisiana sweet crude oil was more toxic than the Peruvian one, probably due to the properties of the oils since the Peruvian crude oil is considered heavy and less toxic compared to light crude oils.

Chapter 3 reported PAH concentrations, EC_{50} obtained from Microtox® Acute Toxicity Test, and mutagenicity of oil-contaminated water and sediment from two areas in the Peruvian Amazon, and Peruvian crude oil. The highest total PAH concentration near Saramuro was found in water, 210.15 µg/ml, and the highest in Trompeteros was also found in water, 204.66 µg/ml. All water samples tested for Saramuro and Trompeteros, and one sediment sample were found to be mutagenic for both strains TA98 and TA100. While, Peruvian crude oil was mutagenic using strain TA98 and S9 enzyme, suggesting that it does not possess direct mutagenic activity and future tests should all include S9 activation.

Results from the Microtox Test showed that on sediment sample was toxic in Saramuro (EC_{50} = 335.1 mg/l), and in Trompeteros toxicity ranged from 25.67 to 133.86 mg/l, one in water and two in sediment samples. The EC_{50} for WAF using Peruvian crude oil was 17.18 mg/l. The two areas sampled had very high PAH concentrations that are most likely associated with oil activities. The results in the present study suggest that even though the water and sediment samples collected contained PAHs, the toxic effects are not acute. However, since most of the samples were mutagenic, it is thought that DNA structure could be damaged. This confirms that wastewater containing oil is comprised of harmful substances; including those with genotoxic and carcinogenic effects, and that the oil industry in Peru has the potential and may be severely degrading aquatic organisms and people's health.

Since bioassays are an important tool used to provide background information for risk assessment of chemicals, other fish species should be tested in the future. Research needs to include a battery of short-term bioassays in order to predict acute toxicity of heavy metals such as cadmium and mercury, which are related to oil activities. Additional assays are recommended in order to determine all the contaminants present in the water and sediment of these rivers. Thus, concentration of the 16 priority PAHs in fish samples from different sites in both rivers should be determined. Microbial, chemical, and additional toxicological tests on more species are necessary to predict effects of oil and implement bioremediation methods to alleviate the damage that oil industry has caused in this part of Peru.

Oil production is still a growing industry in Peru; therefore, it is important to determine the toxic, carcinogenic and mutagenic effects of pollutants related to oil

activities. With a better understanding of the chemical and biological properties of these pollutants, organisms and human populations could be protected.

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APPENDICES

Appendix A. Tables showing the 96 hour-toxicity test of three reference toxicants (zinc sulfate, sodium dodecyl sulfate, Louisiana sweet crude oil), and Peruvian crude oil in replicates on a Peruvian fish species, red pacu *Piaractus brachypomus.* Well water from IIAP (Iquitos, Peru) was used as dilution water and it had 32 mg/l as $CaCO_3$ of alkalinity, 24 mg/l as $CaCO_3$ of hardness, 7.1 pH, and 4.3 mg/l DO.

Toxicant: Zinc sulfate

Dilution water: Well water

Organisms: Red pacu *Piaractus brachypomus*, 14 days old

	24 hr			48 hr			72 hr			96 hr				
Replicates	1	2	3	1	2	3	1	2	3	1	2	3	Total alive	% survival
Control	10	10	10	10	9	10	10	8	10	10	7	10	27	90.0
1.875 mg/l	10	9	10	10	8	10	9	8	10	5	5	9	19	63.3
3.75 mg/l	10	9	10	8	9	9	8	9	9	4	4	7	15	50.0
7.5 mg/l	10	9	10	10	9	9	9	6	8	7	5	2	14	46.7
15 mg/l	9	8	9	4	8	6	1	5	5	0	3	2	5	16.7
30 mg/l	6	6	7	1	4	1	0	1	0	0	0	0	0	0.0

Toxicant: Sodium dodecyl sulfate (SDS)

Dilution water: Well water

Organisms: Red pacu *Piaractus brachypomus*, 16 days old

	24 hr			48 hr			72 hr			96 hr				
Replicates	1	2	3	1	2	3	1	2	3	1	2	3	Total alive	% survival
Control	10	10	10	10	10	9	9	9	9	9	9	9	27	90.0
5 mg/l	9	10	10	9	10	10	6	7	6	5	5	5	15	50.0
10 mg/l	10	9	10	10	8	10	7	5	7	5	5	5	15	50.0
15 mg/l	8	9	8	8	9	8	7	8	6	3	4	3	10	33.3
20 mg/l	5	5	4	5	3	3	3	2	2	3	1	2	6	20.0
25 mg/l	0	0	0	0	0	0	0	0	0	0	0	0	0	0.0

Toxicant: Louisiana sweet crude oil (WAF: 25g/L), 22 hours stabilization
Dilution water: Well water
Organisms: Red pacu *Piaractus brachypomus*, 10 days old

		24 hr			48 hr			72 hr			96 hr				
Replicates		1	2	3	1	2	3	1	2	3	1	2	3	Total alive	% survival
Control		10	10	10	10	10	10	10	10	10	10	10	9	29	96.67
6.25%	0.2 mg TPH/l	10	10	10	9	10	10	8	8	10	8	8	8	24	80.00
12.50%	0.4 mg TPH/l	9	10	10	9	10	10	9	10	10	8	7	10	25	83.33
25%	0.7 mg TPH/l	10	10	10	10	10	10	9	10	9	9	9	7	25	83.33
50%	1.5 mg TPH/l	10	10	10	10	10	10	9	10	9	8	7	8	23	76.67
100%	2.9 mg TPH/l	3	4	7	3	4	7	2	2	3	1	2	3	6	20.00

Toxicant: Iquitos crude oil (WAF: 50g/L), 22 hours stabilization
Dilution water: Well water
Organisms: Red pacu *Piaractus brachypomus*, 11 days old

		24 hr			48 hr			72 hr			96 hr				
Replicates		1	2	3	1	2	3	1	2	3	1	2	3	Total alive	% survival
Control		10	10	10	10	10	10	10	10	10	10	9	9	28	93.33
6.25%	0.3 mg TPH/l	10	10	10	10	10	10	10	9	9	9	8	8	25	83.33
12.50%	0.5 mg TPH/l	10	10	10	10	10	10	9	10	8	8	8	7	23	76.67
25%	1 mg TPH/l	10	10	9	10	10	9	10	9	9	7	6	8	21	70.00
50%	2 mg TPH/l	10	10	9	10	10	9	10	9	9	8	7	5	20	66.67
100%	4 mg TPH/l	9	10	10	9	9	9	9	9	9	5	5	8	18	60.00

Appendix B. Table showing the 96 hour-toxicity of Peruvian crude oil in fathead minnows *Pimephales promelas.* The dilution water used in Troy, AL was aerated tap water, and it had 188 mg/l as $CaCO_3$ of alkalinity, 16 mg/l as $CaCO_3$ of hardness, 8.5 pH, and 7.5 mg/l DO.

Toxicant: Peruvian crude oil (WAF: 200g/L), 22 hours stabilization
Dilution water: Tap water (Troy, AL, U.S.A)
Organisms: Fathead minnows *Pimephales promelas*

		24 hr			48 hr			72 hr			96 hr				
Replicates		1	2	3	1	2	3	1	2	3	1	2	3	Total alive	% survival
Control		10	9	10	10	9	10	10	9	9	10	9	9	28	93.3
6.25%	1 mg TPH/l	10	9	9	8	8	9	7	6	7	6	5	6	17	56.7
12.50%	2 mg TPH/l	9	10	10	8	9	6	6	6	5	6	5	3	14	46.7
25%	4 mg TPH/l	10	9	7	7	7	6	5	5	3	4	3	1	8	26.7
50%	8 mg TPH/l	9	10	9	5	7	7	4	3	6	2	2	3	7	23.3
100%	16 mg TPH/l	10	9	9	6	8	5	4	3	2	1	2	1	4	13.3

Appendix C. Tables showing the 24 and 48 hour-range finding tests of zinc sulfate, sodium dodecyl sulfate (SDS), and Peruvian crude oil (WAF using 50 g/l) using 3 individuals of a Peruvian catfish species, doncella *Pseudoplatystoma fasciatum*. The dilution water used for these tests was obtained from Amazon Tropical Aquarium EIRL, and the water quality was as follows: 36 mg/l as $CaCO_3$ of alkalinity, 28 mg/l as $CaCO_3$ of hardness, 7.2 pH, and 4.8 mg/l DO.

Zinc sulfate	24hr
Control	3
0.1 mg/l	3
0.3 mg/l	2
1 mg/l	3
3 mg/l	3
10 mg/l	0
30 mg/l	0

SDS	24 hr	48 hr
Control	3	3
0.1 mg/l	3	3
0.3 mg/l	3	3
1 mg/l	3	3
3 mg/l	3	3
10 mg/l	2	1
30 mg/l	3	0

Peruvian crude oil	24 hr	48 hr
Control	3	3
6.25%	2	2
12.50%	3	3
25%	3	3
50%	2	1
100%	3	1

Appendix D. Tables showing the 24 hour-range finding test of water and sediment from Marañón River near San José de Saramuro (S1 – S5), and Corrientes River near Villa Trompeteros (T1 – T6) in angel fish *Pterophyllum scalare*. Note: N/A = not available. The dilution water used for these tests was obtained from Amazon Tropical Aquarium EIRL, and the water quality was as follows: 36 mg/l as $CaCO_3$ of alkalinity, 28 mg/l as $CaCO_3$ of hardness, 7.2 pH, and 4.8 mg/l DO.

Water	24 hr
Control	3
S1	3
S2	3
S3	2
S4	3
S5	3
T1	3
T2	3
T3	3
T4	3
T5	3
T6	2

Sediment	24 hr
Control	3
S1	3
S2	3
S3	3
S4	2
S5	2
T1	1
T2	N/A
T3	N/A
T4	3
T5	0
T6	1

Appendix E. Tables showing water quality parameters from five collection sites in Loreto, Peru, sampled during summer 2011. Note: DO = Dissolved oxygen.

Collection sites on the Marañón River near San José de Saramuro in Loreto, Peru.

Water quality parameters	S1	S2	S3	S4	S5
pH (standard units)	8.45	8.41	8.35	8.34	8.21
DO (mg/l)	5.17	5.21	4.84	4.45	5.55
Hardness (mg/l as CaCO3)	55	52	70	71	50
Alkalinity (mg/l as CaCO3)	60	56	75	78	55
Temperature (°C)	26.9	26.9	26.5	26.5	26.9

Collection sites on the Corrientes River near Villa Trompeteros in Loreto, Peru.

Water quality parameters	T1	T3	T4	T5	T6
pH (standard units)	6.92	6.73	6.8	6.2	6.65
DO (mg/l)	5.81	5.14	5.69	4.94	5.9
Hardness (mg/l as CaCO3)	24	36	38	24	20
Alkalinity (mg/l as CaCO3)	40	30	35	20	14
Temperature (°C)	26.7	25.2	25.2	24.4	25.2