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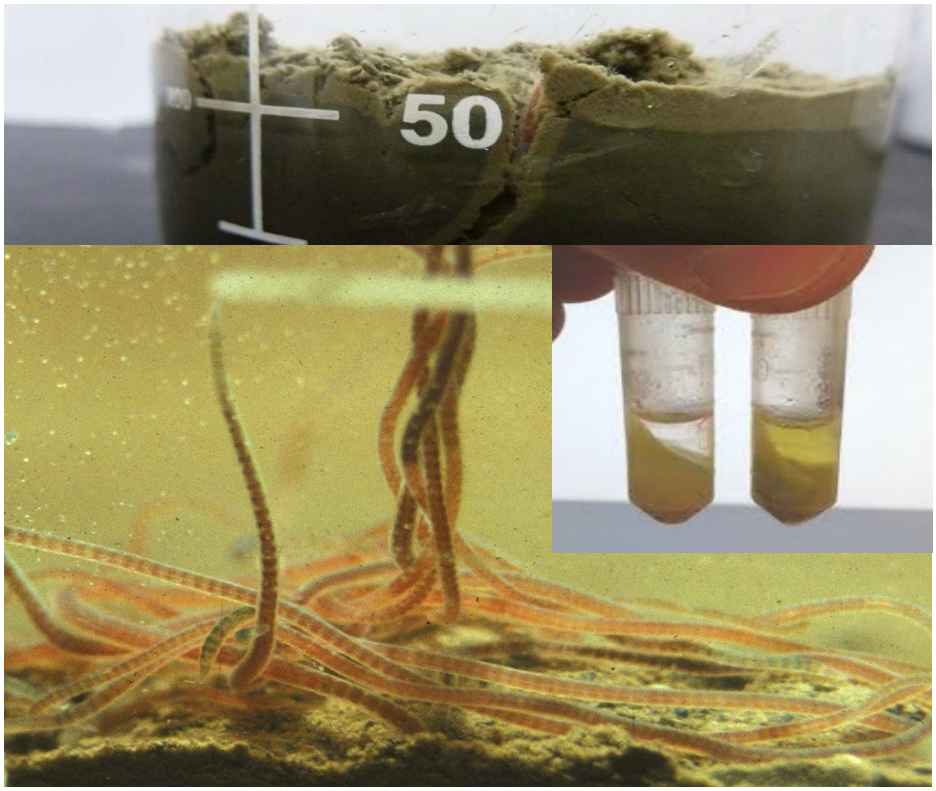
Overview of Microscale Analytical Methods for the Quantitative Detection of Bioaccumulative Contaminants in Small Tissue Masses

Charles H. Laber, Guilherme R. Lotufo, Austin R. Scircle, Jenifer M. Netchaev, and Anthony J. Bednar

February 2024



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Abstract

For many bioaccumulation studies, generation of large sample masses of exposed organisms is challenging or even prohibitive. Therefore, the use of smaller sample masses for analysis without compromising data quality or quantitative level achieved is desirable. To this end, a variety of microanalytical procedures have been developed that used 1 g or less of tissue to address specific experimental challenges. However, these methods have not been systematically evaluated or published. The present work evaluates the current state of the microanalytical methods reported and identifies additional needs that would benefit US Army Corps of Engineers (USACE) research and navigation dredging programs. Discussions with commercial laboratories revealed that they typically do not accept small sample masses and require individual sample masses ranging from 10 to 20 g wet weight of tissue per analysis. If they do analyze a small mass sample, they routinely do not modify their standard process, resulting in detection and reporting limits orders of magnitude higher; therefore, essentially useless nondetect data are generated for regulatory decisions. To address the lack of commercial availability of microanalytical methods, we recommend pursuing method development and subsequent validation of microscale extraction and analysis of a variety of common contaminant compounds in tissue matrices.

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Preface

This study was conducted for the US Army Corps of Engineers (USACE) under the Dredging Operations and Environmental Research (DOER) Program, RT22-022, Funding Account Code U4382272, AMSCO Code 089500, in support of the USACE Operations and Maintenance Navigation Program.

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COL Christian Patterson was commander of ERDC, and Dr. David W. Pittman was the director.

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1 Introduction

1.1 Background

Bioaccumulation is defined as the net accumulation of contaminants from water, diet, and sediment into the tissues of exposed organisms (Weisbrod et al. 2009). Benthic bioaccumulation data can be used to estimate effects in invertebrates directly or support exposure estimates for fish and wildlife that feed on invertebrates (McElroy et al. 2011). In addition, health hazards to humans may ensue from the consumption of shellfish or pelagic fish that have directly or indirectly bioaccumulated sediment-associated contaminants (Gobas and Arnot 2010; Chen et al. 2014). Consequently, assessment of bioaccumulation is commonly included in sediment quality evaluations (USEPA 2016).

Bioaccumulation tests are intended to directly measure the uptake of contaminants into the tissues of the test organism under controlled conditions to provide quantitative information concerning chemical exposure at a contaminated site (Van Geest et al. 2010). Infaunal invertebrates are used as test species and are typically exposed within environmentally controlled conditions in sediment for 28 days. At test termination, organisms are collected from each of the replicate chambers and placed in water for purging of gut content; the organisms' tissues are then homogenized for analytical determination of contaminants of concern (ASTM 2020; test method E1688–19).

Laboratory-cultured oligochaete *Lumbriculus variegatus* (California blackworm) is universally used for evaluation of freshwater sediments (ASTM 2020). The polychaete *Alitta virens* (sandworms; formerly *Nereis virens*) and the marine bivalve *Macoma nasuta* (bent-nosed clam) are the most used test species for marine and estuarine evaluations (ASTM 2020). *Alitta virens* and *M. nasuta* are only available by field collection, and as such, may not be available at the time of experimentation. In addition, natural stressors, including global warming and stress during shipping, may cause those test organisms to become unhealthy, which may lead to bioaccumulation test failure. Field-collected samples may also have background contamination, which is dependent on species and collection environment (Bennett et al. 2011). Laboratory-cultured marine and estuarine invertebrates have been used in sediment bioaccumulation testing. For example, the estuarine benthic amphipod *Leptocheirus*

plumulosus is extensively employed to conduct whole-sediment toxicity experiments (Lotufo et al. 2016) but has also been used to assess bioaccumulation from contaminated sediments (Millward et al. 2005; Bridges et al. 2017; Sinche et al. 2019), and evaluation methods have been developed (Farrar et al. 2011). Laboratory-cultured polychaetes, *Neanthes arenaceodentata*, have also been used in sediment bioaccumulation evaluations (Millward et al. 2005; Janssen et al. 2010, 2011, 2012).

To reduce cost and uncertainty, routine use of bioaccumulation evaluations employing small masses of sediment-dwelling invertebrates, and consequently generating small tissue masses (e.g., 0.5–1 g*) per replicate of laboratory culture, is highly desirable. Such practice is supported by standard guidance for freshwater and sediment bioaccumulation evaluations by ASTM (2020) and USEPA (2000) guidance, which recommend testing in beakers (1 L or smaller) and targeting the harvest of up to 1 g of *Lumbriculus variegatus* tissue per replicate for each chemical analysis (e.g., 1 g for PAHs, 1 g for PCBs, and so forth). In addition, guidance for conducting in situ exposures using caged organisms recommends adding 1–2 g of tissue mass per replicate cage (Burton et al. 2005). Bioaccumulation evaluation studies using standard guidance for invertebrate tissue include Burkhard et al. (2016), who followed USEPA (2000). In that study, 0.25 g of tissue was added to each 300 mL replicate beaker and PCB analysis was conducted on a pool of recovered worms weighing 0.25 g or less (Burkhard et al. 2016). Similarly, Burton et al. (2012) used general guidance from Burton et al. (2005) for in situ bioaccumulation testing, which generated 0.3–1 g of tissue mass for the analysis of PAHs and PCBs.

In addition to sediment bioaccumulation evaluations, benthic bioaccumulation may be assessed by sampling resident infaunal organisms. Collection of benthic invertebrates for bioaccumulation evaluations may yield small masses of organisms for chemical analyses, especially in freshwater environments, where sediment-dwelling invertebrates are typically small and suboptimal compositing of taxa has been necessary (Gobas et al. 1989; Mäenpää et al. 2011; Daley et al. 2011; Abel and Akkanen 2018). Small organism masses are also a concern for

* For a full list of the spelled-out forms of the units of measure used in this document, please refer to *US Government Publishing Office Style Manual*, 31st ed. (Washington, DC: US Government Publishing Office, 2016), 248–52, https://www.govinfo.gov/content/pkg/GPO-STYLEMANUAL-2016/pdf/GPO-STYLEMANUAL-2016.pdf.

marine environments where small macroinvertebrates (e.g., amphipods and corals) or even meiofaunal invertebrates have been sampled for tissue analysis (Ferguson and Chandler 1998; Klosterhaus et al. 2009; Ko et al. 2014; Szczybelski et al. 2016).

Researchers face challenges, such as sample generation, when following standard guidance for freshwater sediment bioaccumulation evaluations (ASTM 2020; USEPA 2000), sediment bioaccumulation testing with estuarine amphipods (Farrar et al. 2011), and in situ sediment bioaccumulation testing (Burton et al. 2005). Researchers collecting small sediment-dwelling invertebrates in the field face challenges when generating samples of sufficient mass for analysis at commercial laboratories. The current analytical methods for fish or aquatic invertebrate tissues employed by commercial laboratories typically require individual sample masses ranging from 10 to 20 g wet weight of tissue per analysis (e.g., 10–20 g each for PAHs, PCBs, organochlorine pesticides, etc.), with additional amounts required for laboratory quality control (Jones et al. 2006) or reanalysis in the case of analytical failure. To achieve the mass requirement of commercial laboratories, researchers must pool individual benthic invertebrates.

Laboratory-cultured invertebrates, including *Lumbriculus variegatus* and *Leptocheirus plumulosus*, typically weigh only 0.005–0.01 g (ASTM 2020; Farrar et al. 2011). Therefore, over 1,000 individual organisms need to be combined to meet the mass requirement for each analysis. In contrast, sediment bioaccumulation evaluation organisms typically used in marine and estuarine dredged material evaluations, *Macoma nasuta* and *Alitta virens*, weigh approximately 3–10 g (Kennedy et al. 2010). Therefore, combining a few individual organisms is sufficient to meet commercial analytical requirements. Compliance with the mass requirements of commercial laboratories means testing in large aquaria using large volumes of sediment. For example, the use of 20–30 L test aquaria for sediment bioaccumulation testing using *Alitta virens* and *Macoma nasuta* is recommended for testing of dredged material (USEPA and USACE 2008).

Chemical analysis of environmental samples generally involves multistep manipulations of the matrix prior to analytical quantitation by laboratory instrumentation. These manipulations often involve extraction of the target analytes from the bulk environmental matrix (e.g., water, sediment, or tissue), transfer to the appropriate (generally liquid) media, removal of

concomitant matrix components and interferents, and finally, analysis by a determinative method. Each of these manipulations directly affect the quality (as assessed by a variety of quality control samples and procedures) and the quantitative level (sensitivity) of the final data. Additionally, the initial starting mass and the final volume analyzed further affect both data quality and quantitative level detectable and are minimally adjustable within constrained standard methods and instrumental limitations.

For many bioaccumulation studies, generation of large sample masses of exposed organisms is challenging or even prohibitive; therefore, use of smaller sample masses for analysis is desirable, without compromise of data quality or quantitative level achieved. To this end, a variety of microanalytical procedures have been developed by various research groups that used 1 g or less of tissue to address specific experimental challenges. Microscale analytical methods for reliable measurement of bioaccumulative contaminants with reduced tissue mass requirements are considered highly advantageous in dredged material evaluations (Farrar et al. 2011). Also, microscale analytical methods would benefit studies involving the measurement of tissue residues of infaunal invertebrates sampled from contaminated sites (Klosterhaus et al. 2009). A typical dredged material evaluation with a target mass of 100 g of tissue per replicate of the sediment bioaccumulation test would require the field crew to collect up to 250 kg of sediment, a labor-intensive event. The use of microscale analytical methods coupled with appropriate bioaccumulation experiments (e.g., using *Lumbriculus variegatus* for freshwater evaluations and *Leptocheirus plumulosus* for marine and estuarine evaluations) is expected to reduce the tissue requirements to 3 g or less, requiring collection of only 2–3 kg of sediment, which is a significant cost savings in labor and experimental space. Therefore, a path toward commercial laboratories providing analysis of small tissue samples as a routine service is highly desirable. For researchers and project managers (e.g., personnel deciding on biological testing for dredged material evaluations) to consider microscale methodologies, these methods must have comparable procedures, equipment, and quantitative limits to the traditional methods and reach regulatorily required detection limits.

1.2 Objectives

The objectives of this special report are to (1) present the results of a literature review of microscale analytical methods for PCBs, PAHs, pesticides, metals, methylmercury, and total lipids; (2) present the survey results of commercial analytical laboratories to gauge their interest and requirements for offering small mass analysis as a routine service; and (3) provide a path forward to expand the use of microscale analytical methods beyond the research laboratory and as a routine service offered by commercial analytical laboratories.

1.3 Approach

This report provides a review of the scientific literature reporting the analysis of small mass samples of aquatic animals, with focus on benthic invertebrates. For the purposes of this report, small tissue sample mass for chemical analyses is defined as 1 g wet weight or less. Therefore, only those studies or methods aimed at analyzing tissue masses of 1 g wet weight or less are considered a microscale analytical method. Throughout the literature, researchers have developed extraction and analytical methods for microgram amounts of tissues from various species. Several studies focused on methodology development for the analysis of small samples for fish or aquatic invertebrates, while other studies present the microscale analytical methodology that was used to support experimental research involving determination of bioaccumulation in aquatic organisms. In this report we highlight studies that analyzed compound groups that are of interests for dredged material evaluations, with a focus on PCBs, PAHs, pesticides, metals, methyl mercury, and lipids.

2 Literature Review: Analysis of Small Mass Aquatic Animal Samples

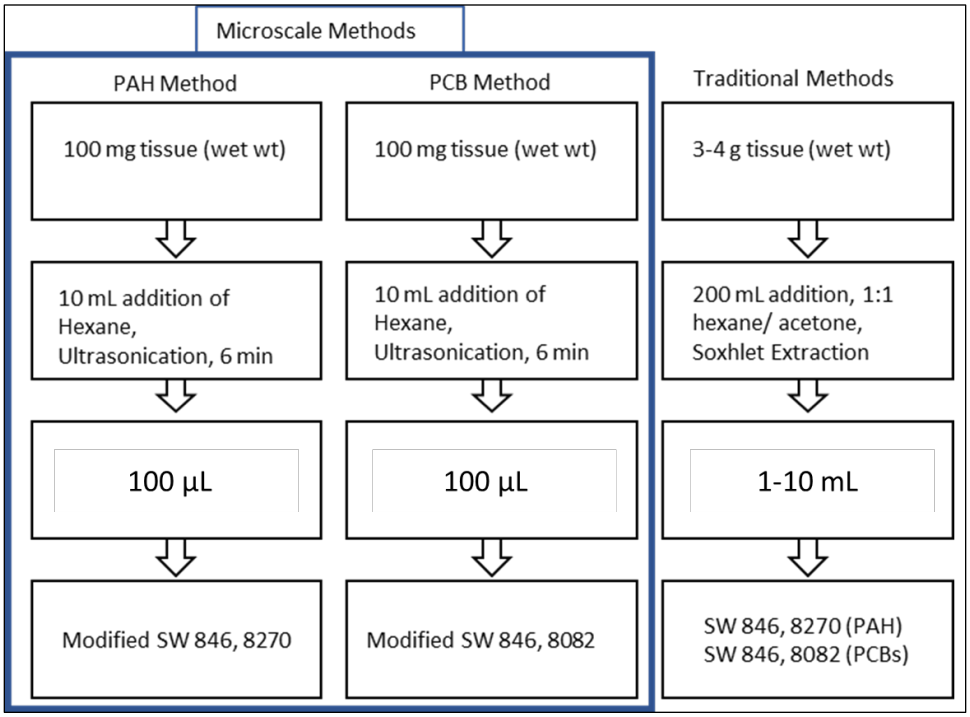
2.1 Polychlorinated Biphenyls (PCBs)—Aroclors and Congeners

Polychlorinated biphenyls have been banned in the United States since the late 1970s; however, these compounds saw use in a wide variety of fields such as lubricants, electronics, refrigerants, and many more. These compounds were released into the environment from accidental spills, from leaks during manufacturing processes, and from their common use between the 1920s and 1979. Because of the major health and environmental issues observed from these compounds, analysis for PCBs is still common, even decades after use has ceased due to their refractory nature and resistance to degradation. A microscale analytical method for the investigation of the bioaccumulation of Aroclor 1254 in meiobenthic copepods was developed by Wirth et al. (1994). That microextraction technique used a bead beater technique to extract analytes from approximately 20 copepods, yielding 25 µg of dry tissue mass, while keeping the extraction volume at 100 µL. Samples were homogenized for 100 s at 3,000 oscillations/min and stored at 4°C. The following day, samples were centrifuged to separate aqueous and organic phases; the organic phase was used for gas chromatography (GC) analysis. The microextraction method by Wirth et al. (1994) was used for the analysis of small samples of benthic invertebrates in subsequent research (DiPinto and Coull 1997; Ferguson and Chandler 1998; Ferguson et al. 2008).

Jones et al. (2006) and Millward et al. (2007) developed microscale methods for small tissue samples (approximately 0.1 g), employing a relatively simple approach that compensates for small sample amounts through concentration of the final extract to 40–100 µL and used in the quantitation of PAHs and PCBs from a 100 mg sample of certified tissues. Samples were subjected to two 3 min cycles of sonication via a microsonication probe with fresh solvent used each cycle. Extracts were then combined, and further column purification was performed prior to analysis. The samples were concentrated to 100 µL or less and stabilized with tricaprin to reduce analyte loss. These extra steps are not required in traditional methods and can therefore be a burden in the form of labor costs, materials, and sample processing time. Jones et al. (2006) and Millward et al. (2007) compared their microscale methods to traditional methods for the analysis of invertebrate tissue samples (Figure 1). Their

microscale analytical method was used for the analysis of PCB congeners in small masses of infauna invertebrates in subsequent research by Rosen et al. (2012), Bridges et al. (2017), and Abel and Akkanen (2018). Jones et al. (2006) determined that the microscale methods developed could provide detection limits similar to traditional methods for PCBs and PAHs, approximately 1.0 and 27.7 µg/kg, respectively.

Figure 1. Schematic comparison of key methodical differences between microscale and traditional methods for PAHs and PCBs, modified from Millward et al. (2007). USEPA Test Methods for Evaluating Solid Waste (SW-846), USEPA 8270, and USEPA 8082 can be found in USEPA (2023, 2018, and 2007a), respectively.



Miniaturized matrix solid-phase dispersion was developed by Campíns-Falcó et al. (2008) for the extraction of PAHs from bivalve samples (0.1 g, dry weight). Clean up and analyte concentration was accomplished by in-tube solid-phase microextraction (SPME). PAH solutions were loaded onto the SPME capillary column by syringe, then 100 µL of deionized water was used to flush out the remaining sample solution. Before separation and detection, the injection valve was rotated, which allowed analytes to desorb from the capillary column into the mobile phase. Samples were analyzed by HPLC with a fluorescence detector. Benefits of Campíns-Falcó et al.'s (2008) method are low mass requirement (e.g., 100 mg), quick preparation time (approximately 10 min per sample), and cost savings on solvents and materials.

Freshwater infaunal invertebrates were collected from freshwater sites and small samples were analyzed for PCB congeners by Mäenpää et al. (2011). Samples underwent extraction via a sonication probe for 2–3 min with 5 mL of acetone and hexane, 1/1 (v/v*). After the sonication procedure, samples were subjected to an additional 20 min of bath sonication. Samples were then centrifuged to separate tissue from solvent, and the process was repeated with 2–3 mL of solvent mixture. The 3 solvent washes were combined and concentrated under nitrogen to a final volume of 2 mL. To remove organic interferents, the authors used concentrated sulfuric acid washes followed by centrifugation to separate the organic solvent and acid layer, which was pipetted and discarded. After the acid washes, nonane was added to the sample organic solvent layer to retain analytes during the final evaporation step. The analytical method was used in subsequent studies measuring PCB bioaccumulation in small samples of *Lumbriculus variegatus* exposed to contaminated sediment in laboratory exposures (Nybom et al. 2015; Abel et al. 2017).

The efficacy of activated carbon addition as a remediation treatment to PCB contaminated sediments was evaluated in a series of studies using laboratory exposures of *L. variegatus* (Sun and Ghosh 2007; 2008, Sun et al. 2009; Beckingham and Ghosh 2010). For those studies, small tissue samples composed of as few as 15 worms, approximately 90 mg, were subjected to PCBs contaminated sediments, 80–100 g, in beakers prior to extraction and analysis. Sun and Ghosh (2008) had a different exposure beaker setup compared to other studies; they began experiments with 80–100 individual *L. variegatus* and each exposure beaker contained 150 mL of site sediment and 100 mL of site water. All authors homogenized and extracted worms by sonication using hexane and acetone, 1/1 (v/v), according to USEPA method 3550B (USEPA 1996a), and extract cleanup was performed according to USEPA methods 3660B (activated Cu) (USEPA 1996c), 3665A (sulfuric acid) (USEPA 1996a), and 3630C (silica gel) (USEPA 1996d). PCB congers analysis was performed by gas chromatography with electron capture detection following modifications of USEPA method 8082A (USEPA 2007a) for these studies.

Small batch bioaccumulation of PCBs in *L. variegatus* from field-collected sediments was evaluated using 300 mL beakers each containing 0.250 g (wet weight) of worms (Burkhard et al. 2013). Tissue was extracted by

* Volume per volume

grinding with excess sodium sulfate and then extracted with hexane for 4 h using a sonication bath. The extracts were prepared for gas analysis using activated silica gel chromatography and concentrations were determined using gas chromatography–mass spectrometry (GC-MS) operated in selected ion monitoring mode. Detection limits for individual congeners were approximately 1 ng/g sediment and 6 µg/g (wet weight) tissue.

In a study of PCB residues in perch eggs, Daley et al. (2009) developed a microscale technique for 0.2–0.5 g sample masses that reflects a miniaturized version of the cold column extraction described in Lazar et al. (1992). Briefly, eggs and larvae were pooled to obtain approximately 0.5 g of sample, which was then homogenized by mortar and pestle with 15 g of activated sodium sulfate. The homogenized samples were then wet packed with 15 mL of methylene chloride and hexane, 1/1 (v/v), into 20 mL glass syringes and gravity filtered through 1 µm glass fiber filter, followed by column extract using three fractions: hexane (discarded), 15%/85% of methylene chloride/hexane, and 50%/5% of methylene chloride/hexane before concentration. Daley et al. (2009) utilized a 50 ng/mL solution of PCB-30, 2,4,6-trichlorobiphenyl (AccuStandard, New Haven, Connecticut) as a surrogate compound to monitor analyte extraction efficiency. The same methodology, with minor modifications, was used in a study of PCB accumulation in 0.2 g samples of field-collected mayflies (Daley et al. 2011).

In pioneer studies of the remedial efficacy of activated carbon amendments to contaminated sediment, Millward et al. (2005) and Zimmerman et al. (2005) were two of the first studies to use small infaunal invertebrates in a sediment bioaccumulation assessment. The bioaccumulation of PCBs in *Leptocheirus plumulosus* and *Neanthes arenaceodentata* were assessed in 1 L beakers, each loaded with invertebrate mass equivalent to approximately 0.1–0.15 g (wet weight). PCB concentrations in tissues were analyzed using standard USEPA methods scaled to use small masses of tissue. For the quantitation of PCBs, amphipod samples were sonicated using USEPA method 3550C (USEPA 2007b), modified by using 10 mL hexane and sonicating the sample at 50% pulse for 6 min using a microtip probe. Methods based on USEPA 3630C (USEPA 1996d) were used for cleaning and solvent evaporation, with extracts reduced to 40 µL. PCB congeners were analyzed using USEPA 8270 (1996e) and selective-ion monitoring GC-MS with detection limit <0.25 µg/kg wet weight. Related follow up studies (Janssen

et al. 2010, 2011) used the extraction methodology described in Millward et al. (2005) for the analysis of small masses of *N. arenaceodentata*.

The bioaccumulation of sediment-associated PCBs in *Leptocheirus plumulosus* using the standard method by Farrar et al. (2011) was also evaluated by Sinche et al. (2019). Small tissue samples (i.e., samples of up to 50 amphipods with estimated mass of approximately 0.2 g) were extracted and analyzed as described in Sinche et al. (2019, p. 1191): "Amphipods were extracted using a high-intensity probe sonicator (Qsonica) for 20 s (Trimble et al. 2008). First, the scintillation vials with frozen amphipods were thawed and spiked with the 2 surrogates DBOFB [dibromooctafluorobiphenyl] and PCB-186 (50 ng each) prior to tissue extraction to determine the tissue extraction performance. Next, 10 mL of acetone was added to vials and homogenized using the probe sonicator. The sonication step was repeated 2 additional times or until the tissues were completely ground. Next, 10 mL of hexane was added to the vials, followed by bath sonication for 10 min. The tissue extracts were then filtered through anhydrous Na2SO4–packed columns (1 g) to remove tissue debris and any remaining water. The filtered extracts were then concentrated under a nitrogen stream to 2 mL, exchanged into 10 mL of hexane, and concentrated to 2 mL prior to acid cleanup. The clean extracts were solvent exchanged and transferred to gas chromatography vials for final chemical analysis."

From the highlighted studies previously described, researchers have been developing or modifying extraction and analytical methods to use small sample masses for decades, specifically for PCB analysis. Our review of these methods illustrates that microextraction and analysis is reproduceable and reliable for PCB Aroclors and congeners. Therefore, microscale methodologies should be further standardized for routine analysis as a cost savings step without fear of reducing data quality.

2.2 Polycyclic Aromatic Hydrocarbons (PAHs)

PAHs are a natural occurring class of chemicals that are a concern in the US due to their carcinogenic properties, which leads them to be a major analysis in dredge chemical prescreening. PAHs are commonly found in crude oil, gasoline, and many other items that are used daily around the world. They make their way into US waterways by accidental spillage and other pathways, making these compounds important to monitor. The

benefits of these extraction and analytical methods for PAHs are similar to those mentioned for PCBs compounds. However, some PAHs can be more volatile than PCBs, which can add challenges to concentration and cleanup procedures. Various extraction and analytical methods for PAHs have been developed and reported in the research literature. The chosen publications for our discussion primarily focus on various tissues; however, some include sediments or other solid materials (Ko et al. 2014).

The accumulation of PAHs in the oligochaete *Ilyodrilus templetoni* from field-collected sediments was evaluated in 50 mL glass tubes loaded with 50 g of sediment and 15 worms (estimated mass of approximately 0.1–0.15 mg wet weight) (Lu et al. 2006). Sediment and tissue samples were extracted by an ultrasonic method based on the USEPA method 3550C (USEPA 2007b). Accumulation of PAHs in infaunal invertebrates was investigated using 60 mL glass jars containing 85 g of sediment and loaded with invertebrate masses ranging from approximately 0.3 to 1 g (Rust et al. 2004). While Rust et al. (2004) investigated sediment and tissues in their study, we will focus on the tissue extract procedures of Ko et al. (2014) for the following discussion. To evaluate the accumulation of PAHs in corals from a contaminated site, pieces of coral were sonicated in a mixture of methylene chloride and methanol 1/1 (v/v) extraction solvent for 10 min to enhance the release of analytes from coral coenosarcus into the extractant. After Soxhlet extraction for 24 h, the unbroken (same shape) skeletal carbonate of each sample was baked for 8 h at 60°C and weighed. The mass of the analyzed coral tissue was determined by calculating the difference between the weights of the whole sample before and after extraction (0.1–0.7 g) (Ko et al. 2014). Cleanup of the extract from sediment and coral samples was performed on an alumina trioxide column using acid-treated and activated silica gels. The extractant was used to elute all analytes, concentrated to about 5 mL using a rotary evaporator (Panchum Inc.), and then evaporated to a final volume of about 0.5 mL using a gentle stream of nitrogen. The eluent fractions containing a suite of PAHs were analyzed using high resolution capillary GC and mass spectrometry (MS) (Ko et al. 2014).

Ko et al. (2014) analyzed PAHs in coral and sediment from the coast of Taiwan. For sediment extractions, researchers ground approximately 1 g with anhydrous sodium sulfate and used an accelerated solvent extraction (ASE) 300 extractor (Dionex, Thermo Fisher Scientific, Waltham, Massachusetts). Sample extracts were concentrated to 0.5 mL under

gentle nitrogen flow before analysis. A GC-MS with ion trap detector was used for analysis with a constant flow of 2 mL/min of carrier gas, helium. The researchers used a 30 m DB-5 column, 0.25 mm inner diameter, 0.25 mL, to analyze for 39 semivolatile organic compounds.

Wirth et al. (1994) developed a microextraction method that was later used by Klosterhaus et al. (2009) for the extraction of PAHs from small masses (55–80 µg dry weight) of meiobenthic copepods. The extracts were analyzed by a GC that was paired with a 5% phenyl methyl-silicone capillary column, 25 m. Sample injection was performed in splitless mode with helium carrier gas at a rate of 1 mL/min at an injector temperature of 200°C. The oven ramp protocol consisted of an initial temperature of 80°C, then ramped to 185°C by 10°C/min, followed by a 5°C/min ramp from 185°C to 270°C, which was held for 15 min. The last ramp was 20°C/min to the final temperature of 300°C and held for 10 min (Klosterhaus et al. 2009).

Researchers have also performed comparative studies with Atlantic cod and haddock eggs to determine whether solid-phase extraction (SPE) and mixed phase dispersive SPE would have less coelution of lipids, and which would prove lower limits of detection of PAHs compared to more traditional extraction procedures (Sørensen et al. 2016). Twenty-seven compounds were spiked into tissue within the concentration range of 10–1,000 ng/mL. 100 mg of cod or haddock eggs underwent a liquid sonication extraction with a mixture of dichloromethane and n-hexane (1:1, 1:9, and 9:1 [v/v]) followed by centrifugation. The supernatant was removed and the process was repeated with fresh solvent; and all supernatant was combined and concentrated under nitrogen gas before sample purification. Sørensen et al. (2016) performed SPE with three different sorbents, silica, Florisil, and alumina, at a mass of 500 mg and eluted with 6 mL of solvent, 0%–50% dichloromethane in n-hexane. After purification, sample extracts were concentrated to 0.5 mL for analysis on a GC-MS in select ion moderating mode. The conclusion of that work was that the SPE method allowed for an order of magnitude lower limits of detection compared to traditional methods that use more sample mass and more complicated extraction and purification procedures.

PAHs are a major analyte group for predredging chemical analysis performed alongside many US Army Corps of Engineers (USACE) dredging operations. However, that analysis suffers from issues due to the

inconsistency of requirements from the USEPA regions and their various limits of detection for analytes. A multitude of researchers have performed analyses on 0.5 g or less by using purification of liquid extractions and varying solid mass and extract volume (Martinez et al. 2004; Pena et al. 2006; Navarro et al. 2009; Sanz-Landaluze et al. 2006). In general, utilizing a microsonication probe appears to be a universal technique for samples under 0.5 g in mass compared to other instrumental techniques, such as ASE, or the traditional Soxhlet procedure. Based on our literature review, analysis of PAHs can be miniaturized in two ways, initial sample mass and final extract volume. In Section 4, we postulate how the US Army Engineer Research and Development Center (ERDC) would benefit from that miniaturization by updating in-house extraction and purification methods to use fewer initial materials and solvent volumes, and to modernize purification with SPE methods. Other improvements could be the utilization of new instrumentation, such as gas chromatography–tandem mass spectrometry (GC-MS/MS) to further improve analysis and reporting limits.

2.3 Pesticides (Organochlorine and Organophosphorus)

The US uses approximately 1 billion pounds of conventional pesticides annually for control of insects, weeds, and other pests (Alavanjia 2009). Through routine use, runoff of residual compounds can lead to surface or groundwater contamination, as well as contamination of soils and sediments. Many of these compounds are refractory in nature and known to bioaccumulate. Because these compounds are often hydrophobic, they can partition to sediments and therefore be major analytes of interest prior to dredging operations (Lushchak et al. 2018).

Since the early 2000s, the literature has seen an abundance of publications focus on bioavailability of pesticides from sediments. Ingersoll et al. (2003) evaluated the biological uptake of DDT and metabolites from field-collected sediments over a 56-day period. *Lumbriculus variegatus* masses used ranged from 0.53–0.96 g for pesticide analysis of worms exposed to sediments (Ingersoll et al. 2003). Samples were homogenized, mixed with sodium sulfate, extracted by methylene chloride, then prepared for analysis by GC-MS in select ion mode (Ingersoll et al. 2003). Previously, Wade et al. (2002) assessed the bioavailability of DDT in the Little Sunflower River and surrounding agricultural soil in the Mississippi Delta. Researchers initiated

bioaccumulation studies with 1 g of *L. variegatus* and then performed chemical analyses on the surviving worms. For the pesticide analysis, individual whole *L. variegatus* were separated and prepared for gas chromatography–electron capture detector (GC-ECD) analysis (Wade et al. 2002). The results demonstrated tissue concentrations for DDT and metabolites averaging 668.4 µg/kg of wet weight (Wade et al. 2002).

Tse et al. (2004) analyzed for 13 organophosphate insecticides in water, sediment, and trout tissue. Researchers homogenized 10 g of tissue spiked with organophosphate compounds, which was then mixed with 6 g of sodium sulfate and equilibrated for 2 hr. Then samples were extracted with 3 aliquots of 100 mL of hexane:acetone, 80:20, and the extracts were combined then concentrated to 1 mL for GC-ECD and as chromatography nitrogen phosphorous detection analyses (Tse et al. 2004). Ackerman et al. (2008) reported on the concentrations of organic contaminants, such as pesticides, PCBs, and PAHs, in fish from Western US National Parks. Larger sample masses, approximately 20 g, were used to have a better assessment of piscivorous wildlife exposure. Samples were extracted with pressurized solvent then extracts were purified by gel permeation chromatography before GC-MS analysis (Ackerman et al. 2008). Water, sediment, and tissues were collected from ponds in eastern France by Lazartigues et al. (2011) to analyze for pesticide residues. Researchers mixed 3 g of fish tissue with manganese sulfate then performed a high-performance dispersive extraction with 10 mL of acetonitrile/water, 50:50 v/v (Lazartigues et al. 2011). Samples were then centrifuged, and the liquid portion pipetted into a glass tube. The solids were subjected to a secondary extraction with ethyl acetate/cyclohexane at 75:25 v/v, and then centrifuged again (Lazartigues et al. 2011). The extracts were combined and dried under nitrogen and reconstituted in 900 µL acetonitrile/water, 10:90 v/v, then analyzed by GC-MS/MS (Lazartigues et al. 2011). Other researchers, such as Shrivas and Wu (2008), investigated microextraction using minimal solvent; 1 g of fish tissue was ground and extracted with 1 mL of methanol via sonication then centrifuged and the supernatant diluted with deionized water and filtered (Shrivas and Wu 2008). Researchers used single drop microextraction for analysis, which was performed by 1 µL of toluene being drawn into a 10 µL syringe, then 0.6 µL of the extraction was injected onto the GC-MS (Shrivas and Wu 2008). This specific type of analysis has been unique to pesticide analyses as reviewed during our literature search. The benefits associated with single drop methods include small initial mass required and less expense in

supplies and labor; this type of analysis could be a path forward for microscale analytical methods.

2.4 Metals

The current standard method for metals digestion, USEPA method 3050B (USEPA 1996b), describes acid digestion of 1–2 g of dry sediment or tissue. Therefore, the standard method does not require substantially more material than our original definition of a microscale analytical method. Typical studies of metals in tissue may meet the definition of micromethod depending on their project needs (Rodrigues et al. 2008; Orr et al. 2018). As with modification of other methods previously described, simply reducing the digested mass will result in elevated quantitative limits, and reduction of final digestate volume can introduce analytical interferences. Therefore, substantial method development is needed for low mass samples if the quality of the data and final reporting limits are not changed. These suggested method modifications could also include changes to the analytical methods, such as use of graphite furnace atomic absorption spectroscopy, if limited numbers of analytes are required because of the small volume of digestate used for analysis as compared to inductively coupled plasma techniques. Alternatively, laser ablation inductively coupled plasma mass spectrometry (LA-ICPMS) requires significantly less sample mass; however, since digestion of tissues is not required, the exact mass used is unrecorded. LA-ICPMS has been routinely used for spatial studies of metals distribution in tissues and studies have addressed the challenge of quantitation through multiple approaches (Becker et al. 2005; Austin et al. 2010; and Pugh et al. 2011).

2.5 Methylmercury

Methylmercury (MeHg)* is another common target analyte for USACE chemical determination for dredged material evaluation projects. Methylmercury can be a major issue for environmental health because it is known for bioaccumulation in fish and shellfish, and it is of major concern for its effects on vascular and endocrine systems. In the present work, we

* For a full list of the spelled-out forms of the chemical elements used in this document, please refer to *US Government Publishing Office Style Manual*, 31st ed. (Washington, DC: US Government Publishing Office, 2016), 265, https://www.govinfo.gov/content/pkg/GPO-STYLEMANUAL-2016/pdf/GPO-STYLEMANUAL-2016.pdf.

highlight literature that was performed at the microscale or has comparable detection limits.

A microscale analytical method for methylmercury and labile Hg^{2+} in small fish tissue samples (0.1–0.2 g) was developed by Cai and Boyna (1995). Their analytical procedure involves aqueous-phase derivatization of ionic mercury species with sodium tetraethylborate in a sample vial and subsequent extraction with a SPME fiber with polydimethylsiloxane coating. Fiber deployment was either to the headspace SPME or to the aqueous sample. The mercury derivatives are quantified by electron impact mass spectrometry.

Amirbahman et al. (2013) compared extraction and detection for total mercury and MeHg in the matrices of pore water, sediments, biota (*Leptocheirus plumulosus*, *Alitta virens*, and *Macoma nasuta*), and diffusive gradient in thin films (DGTs). Tissue samples of *L. plumulosus* have an estimated mass of up to approximately 0.1 g. The extraction method was completed following USEPA method 1631 (USEPA 2002) and used a Tekran cold vapor atomic fluorescence spectrometer for analysis. Researchers were able to achieve a detection limit of 1.5 ng/g for THg and MeHg with an initial mass of 0.1 g (Amirbahman et al. 2013).

Buckman et al. (2019) investigated the fate and mobility of MeHg from soils to amphipods and oysters based on the effects of organic carbon percentage and soil temperature. They analyzed pooled 60 amphipods per analytical sample (estimated mass of approximately 0.2–0.25 g) and determined the MeHg and inorganic Hg (iHg) by a commercial laboratory that provides species-specific isotopes measured using two inductively coupled plasma mass spectrometry (ICP-MS) instruments. Their methodology differs from USEPA method 1630 (USEPA 1998) but was verified using certified reference materials from National Institute of Standards and Technology (NIST) with recoveries ranging from 103%–120% for mussels and oysters. Taylor et al. (2019) also used amphipods for their work, which analyzed for MeHg and iHg and used $MeHg^{201}$ and Hg^{199} as internal standards (ISs) during analysis. For the amphipods, researchers freeze-dried the tissue samples after addition of spikes and ISs. Then amphipods were suspended in solution and extracted with a polymer solution, porewater, ultrapure water, and 3 M citrate buffer. Samples were analyzed by purge and trap gas chromatography coupled with an ICP-MS. Curtis et al. (2019) performed MeHg analysis on

amphipods with masses ranging from 0.0016–0.22 g by also using a freeze-drying process. Their method achieved a detection limit of 1.0 ng/g for amphipods and 0.1 ng/g for sediments. Curtis et al. (2019) used a purge and trap gas chromatograph coupled to an ICP-MS similar to that used by Taylor et al. (2019). The difference between their work was that Curtis et al. (2019) used tetramethylammonium hydroxide in the extraction process.

2.6 Total Lipids

Lipids are the major compartment for the partitioning of neutral organic chemicals in animal tissues, and lipid normalization is used to decrease variability in bioaccumulation assessments because lipid content of the exposed organisms influences estimates of tissue concentrations (Burkhard et al. 2003; Randall et al. 1991). The most used standard lipid method is the Bligh–Dyer gravimetric method (Bligh and Dyer 1959). However, the Bligh–Dyer method is considered unreliable for sample sizes below 5 g (Honeycutt et al. 1995), and therefore requires pooling many small aquatic invertebrates, such as *L. variegatus*, for quantification of total lipids content. Methodology that requires only milligram or even microgram quantities of animal tissue have been develop for estimating lipid content of aquatic biota.

Three methodologies have been used for quantifying total lipids in small samples of fish and aquatic invertebrates. Gardner et al. (1985) developed a microgravimetric method modified from Bligh and Dyer (1959) to measure total lipids in freshwater aquatic invertebrates. Parrish (1987) adapted a microquantity thin-layer chromatographic approach, using an Iatroscan flame ionization detection system to measure lipid classes, with total lipids reported as the sum of the lipid classes, for use with aquatic invertebrates. The third methodology uses the colorimetric sulfo-phospho-vanillin (SPV) method developed by Chabrol and Charonnat (1937) to measure total lipids in serum as adapted by Van Handel (1985) for measuring the lipid content of a single mosquito.

The microgravimetric method by Gardner et al. (1985) has been used with benthic invertebrates and zooplankton (e.g., Landrum 1988; Vanderploeg et al. 1992; Leppänen and Kukkonen 1998), and the thin-layer chromatographic approach by Parrish (1987) has been used with zooplankton, benthic macroinvertebrates, larval fish, and juvenile fish (Lu

et al. 2008). The method by Gardner et al. (1985) has been considered time and labor intensive compared to the colorimetric method by Van Handel (1985) (Randall et al. 1991; Inouye and Lotufo 2006; Lu et al. 2008) and has therefore been used less frequently. Although the micro-colorimetric SPV method (Van Handel 1985) has been preferred over the microgravimetric method (Gardner et al. 1985) for measuring total lipids of invertebrates because it is more time and labor efficient (Landrum et al. 2002, 2004; Inouye and Lotufo 2006), a comprehensive comparison of the two microquantity methods has not been conducted for any organism. The Iatroscan thin-layer chromatographic–flame ionization detection (TLC–FID) method sums individual lipid classes to generate an estimate of total lipids, and therefore, has the potential to underestimate lipids (Lu et al. 2008). The Iatroscan TLC–FID method has also been used less frequently compared to the microcolorimetric SPV for total lipids determination. For aquatic biota, total lipids determination using the microcolorimetric SPV method were not statistically different from gravimetric analysis for aquatic biota (Landrum et al. 2002; Inouye and Lotufo 2006; Lu et al. 2008; Pinger et al. 2022). The microcolorimetric SPV has been adapted for rapid and high throughput quantitation using microplates (Cheng et al. 2011; Pinger et al. 2022) and adapted for measuring lipids in copepod samples as small as 50 μg dry weight (Klosterhaus et al. 2009).

3 Results of Inquiries to Commercial Analytical Laboratories

We contacted 10 of the ERDC Environmental Chemistry Branch's contract laboratories and one nonprofit laboratory, Battelle, to gauge current interest and use of microextraction methods in the industry. Eight of the laboratories did not respond to our multiple attempts for this initial discussion, only two contract laboratories and Battelle were willing to have a phone conversation, and none responded by email. Our questionnaire sent to the laboratories focused on three topics: (1) what analyses could use 0.5 g and the associated reporting limit, (2) what mass is required for their methods, and (3) what is their capability and willingness to use modified analytical techniques for small mass samples. The discussions that occurred with the two contract laboratories that responded revealed little interest in using or developing small mass extraction procedures for tissues analysis. The contract laboratories stated that they were not equipped to provide analysis of small tissue samples or that it was not financially feasible to have these capabilities in-house because these samples would be rare compared to their normal workload. The laboratories also stated that they rarely, if at all, deviate from the methods for which they are certified. For example, if a laboratory is certified for a PAH analysis that requires 10 g per sample, the laboratory will either reject or use your (less than required) sample but will not alter the rest of the sample preparation, purification, or concentration steps from the procedure for 10 g samples, which dramatically increases the final reporting limit.

For the "not equipped" response, laboratories argued that tissue methods require some instrumentation for purification protocols that are expensive. For example, USEPA method 3640A (USEPA 1994) requires a gel permeation chromatograph (GPC), frequently used to remove lipid content from organic extractions prior to further clean up procedures and analysis, that costs approximately $100,000. Maintenance and daily operational cost to have the GPC capability can also be expensive with the liters of solvents, instrument parts, and labor. Expounding on that laboratories do not find tissue analysis economical, most discuss that the high startup cost does not include the labor cost of training current personnel or finding skilled experienced employees for tissue projects. Many of the contract laboratories mentioned that most clients do not

typically submit enough tissue samples for analysis (typically less than 20 samples), so they are unable to justify the costs when they can sub-contract with a specialty laboratory that handles strictly tissue analyses.

During our conversations with commercial laboratories, certifications and lack of standard methods for tissue matrices were a significant component for lack of interest. Many of these companies have spent a significant amount of time and money to become certified through Department of Defense Environmental Laboratory Accreditation Program and/or The National Environmental Laboratory Accreditation Conference (NELAC) Institute's (TNI) National Environmental Laboratory Accreditation Program (NELAP). However, the US Department of Defense Quality Systems Manual (US DoD 2019) does not contain a tissue matrix section; instead, tissues are consolidated into a category of solids, which does not account for the extraction or analytical challenges associated with tissue matrices. The number of TNI certified laboratories for tissue matrices is small compared to those certified for water matrices. For example, USEPA method 8270E (USEPA 2018), semivolatile organics compounds by GC-MS has 10 certified laboratories for tissues matrices compared to 207 certified laboratories for water; this trend is consistent across multiple analyses. Because of the lack of standardized methodologies and few certified laboratories for small mass samples, USACE districts would face challenges if opting for generating small mass tissue samples during environmental projects, including dredged material evaluations. Many USACE districts are being required by stakeholders to use certified laboratories, yet these commercial entities require large sample masses for the standardized methods for which they are certified. Modification of these methods for lower reporting limits, or to apply to small mass samples, is outside their certification; and therefore, data generated is often insufficient to support many environmental projects.

4 Conclusions and Path Forward

Microscale analytical methods for analysis of biota are relevant for (1) experimental research projects that are resource limited (generating large masses can be expensive) and use small organisms (e.g., copepods); (2) field investigations involving sampling of small organisms (e.g., amphipods and aquatic oligochaetes); and (3) sediment bioaccumulation tests such as those performed using standard guidance (ASTM 2020; USEPA and USACE 1991, 1998). These requirements are not met by many standard analytical protocols developed by USEPA and other entities, because those methods generally require 1–10 g of sample per analysis.

Based on the extensive literature search for microscale analytical methods and the responses we received from commercial laboratories, there is clear divide in desire for these methods; research facilities have developed many variations of microscale analytical methods, generally adapted to a specific research experimental setting. These modified methods are not always directly applicable to other complex or challenging research scenarios, leading to "modifications" of modified methodologies—and ever-increasing analytical uncertainty. In comparison, many commercial laboratories are primarily concerned with economic viability and adherence to method protocols that they are certified to perform. There is also a reticence to stray beyond standard protocols because of unknown analytical challenges that may present themselves, which would again lead to analytical uncertainty. This void provides a path for ERDC to explore and create new research efforts to span these disparate goals.

ERDC has previously demonstrated the ability to develop and even validate analytical methods and transition those to USEPA. A clear example is the analysis of explosives residues using USEPA method 8330A (USEPA 2007c), for which the original methodology was developed by researchers at ERDC's Cold Regions Research and Engineering Laboratory (CRREL) and then further modified to include multi-increment composite sampling (USEPA method 8330B) (USEPA 2006). Most recently, ERDC Environmental Laboratory (EL) researchers have added insensitive munitions to the analyte list and are working with USEPA to include this process as an addendum to Method 8330B (Scircle et al. 2023; Crouch et al. 2020; Russell et al. 2014). This research and subsequent administrative tasks are executed over the course of many years, often approaching a decade, with associated costs of several million dollars. The results,

however, are clearly beneficial to the environmental testing community, such that ERDC is viewed as a leading expert in the field.

To address the limited analytical procedures for microscale analytical procedures, the same level of effort should be put forward as previously discussed for explosives and insensitive munitions analytical procedures. Robust, adequately funded, and time allotted research projects (e.g., $500,000/year for 5 years) should be initiated to specifically develop and validate analytical methods for all target classes of compounds (e.g., PCBs, pesticides, and metals) of interest to the USACE Civil Works program via round robin studies modeled after those recently performed for insensitive munitions constituents (Crouch et al. 2020; Scircle et al. 2023). The transition of new and validated methods to USEPA (Lotufo et al. 2022) and other stakeholders would immediately provide acceptance by the larger scientific and regulatory communities and further demonstrate ERDC's leadership in this area. The investment in the development and validation of microscale analytical procedures is expected to result in tangible benefits for USACE. Coupling of small volume bioaccumulation testing with microscale analytical procedures is expected to significantly improve logistics, maintain quality, reduce costs, and streamline and improve efficiency of the required overall biological testing program for dredged evaluation. The bioaccumulation testing and analysis component of dredged material evaluations may cost up to $100,000 (2023 dollars). The use of miniaturized testing and analysis may decrease the cost by as much as 20%. Compounded across multiple harbors, projects, and USACE districts and divisions, significant return on investment of this R&D would be realized within 5–6 years. In addition to the decrease in cost from miniaturization of sediment bioaccumulation tests, small laboratory-cultured estuarine invertebrates (e.g., *Leptocheirus plumulosus*) can be used in miniaturized testing and analysis, which is highly desirable. Currently, field-collected invertebrates are used in marine and estuarine dredged material evaluations, which is problematic since their availability may be limited at the time of experimentation and variability in native condition or stress incurred during collection and shipping may lead to varying levels of responsiveness.

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Abbreviations

ASE	Accelerated solvent extraction
ASTM	American Society for Testing and Materials
CRREL	Cold Regions Research and Engineering Laboratory
DGTs	Diffusive gradient in thin films
EL	Environmental Laboratory
ERDC	Engineer Research and Development Center
GC	Gas chromatography
GC-ECD	Gas chromatography–electron capture detector
GC-MS	Gas chromatography–mass spectrometry
GC-MS/MS	Gas chromatography–tandem mass spectrometry
GC-NPD	Chromatography nitrogen phosphorous detection
GPC	Gel permeation chromatograph
ICP-MS	Inductively coupled plasma mass spectrometry
iHg	Inorganic mercury
IS	Internal standard
LA-ICPMS	Laser ablation inductively coupled plasma mass spectrometry
MS	Mass spectrometry
NELAC	National Environmental Laboratory Accreditation Conference
NELAP	National Environmental Laboratory Accreditation Program
NIST	National Institute of Standards and Technology

SPE	Solid-phase extraction
SPME	Solid-phase microextraction
SPV	Sulfo-phospho-vanillin
TLC–FID	Thin-layer chromatographic–flame ionization detection
TNI	The NELAC Institute
USACE	US Army Corps of Engineers
USEPA	US Environmental Protection Agency
v/v	Volume per volume

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14. ABSTRACT

For many bioaccumulation studies, generation of large sample masses of exposed organisms is challenging or even prohibitive. Therefore, the use of smaller sample masses for analysis without compromising data quality or quantitative level achieved is desirable. To this end, a variety of microanalytical procedures have been developed that used 1 g or less of tissue to address specific experimental challenges. However, these methods have not been systematically evaluated or published. The present work evaluates the current state of the microanalytical methods reported and identifies additional needs that would benefit US Army Corps of Engineers (USACE) research and navigation dredging programs. Discussions with commercial laboratories revealed that they typically do not accept small sample masses and require individual sample masses ranging from 10 to 20 g wet weight of tissue per analysis. If they do analyze a small mass sample, they routinely do not modify their standard process, resulting in detection and reporting limits orders of magnitude higher; therefore, essentially useless nondetect data are generated for regulatory decisions. To address the lack of commercial availability of microanalytical methods, we recommend pursuing method development and subsequent validation of microscale extraction and analysis of a variety of common contaminant compounds in tissue matrices.

15. SUBJECT TERMS

Chemical agents (Munitions); Explosives--Environmental aspects; Environmental monitoring; Bioaccumulation; Tissues--Testing; Testing--Methodology

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