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Publication Date

2015-07-24

DOI

10.3791/52558

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Video Article

Use of Chironomidae (Diptera) Surface-Floating Pupal Exuviae as a Rapid Bioassessment Protocol for Water Bodies

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URL: http://www.jove.com/video/52558
DOI: doi:10.3791/52558

Keywords: Environmental Sciences, Issue 101, biological monitoring, aquatic systems, aquatic ecology, water quality, macroinvertebrates, midge, Chironomid Pupal Exuviae Technique, rapid bioassessment protocol

Date Published: 7/24/2015

Citation: Kranzfelder, P., Anderson, A.M., Egan, A.T., Mazack, J.E., Bouchard,, R.W., Rufer, M.M., Ferrington,, L.C. Use of Chironomidae (Diptera) Surface-Floating Pupal Exuviae as a Rapid Bioassessment Protocol for Water Bodies. *J. Vis. Exp.* (101), e52558, doi:10.3791/52558 (2015).

Abstract

Rapid bioassessment protocols using benthic macroinvertebrate assemblages have been successfully used to assess human impacts on water quality. Unfortunately, traditional benthic larval sampling methods, such as the dip-net, can be time-consuming and expensive. An alternative protocol involves collection of Chironomidae surface-floating pupal exuviae (SFPE). Chironomidae is a species-rich family of flies (Diptera) whose immature stages typically occur in aquatic habitats. Adult chironomids emerge from the water, leaving their pupal skins, or exuviae, floating on the water's surface. Exuviae often accumulate along banks or behind obstructions by action of the wind or water current, where they can be collected to assess chironomid diversity and richness. Chironomids can be used as important biological indicators, since some species are more tolerant to pollution than others. Therefore, the relative abundance and species composition of collected SFPE reflect changes in water quality. Here, methods associated with field collection, laboratory processing, slide mounting, and identification of chironomid SFPE are described in detail. Advantages of the SFPE method include minimal disturbance at a sampling area, efficient and economical sample collection and laboratory processing, ease of identification, applicability in nearly all aquatic environments, and a potentially more sensitive measure of ecosystem stress. Limitations include the inability to determine larval microhabitat use and inability to identify pupal exuviae to species if they have not been associated with adult males.

Video Link

The video component of this article can be found at http://www.jove.com/video/52558/

Introduction

Biological monitoring programs, which use living organisms to evaluate environmental health, are often used to assess water quality or monitor success of ecosystem restoration programs. Rapid bioassessment protocols (RBP) using benthic macroinvertebrate assemblages have been popular among state water resource agencies since 1989[1]. Traditional methods of sampling benthic macroinvertebrates for RBPs, such as the dip-net, Surber sampler, and Hess sampler[2], can be time-consuming, expensive, and may only measure assemblages from a particular microhabitat[3]. An efficient, alternative RBP for generating biological information about a particular water body involves collection of Chironomidae surface-floating pupal exuviae (SFPE)[3].

The Chironomidae (Insecta: Diptera), commonly known as non-biting midges, are holometabolous flies that typically occur in aquatic environments before emerging as adults on the water's surface. The chironomid family is species-rich, with approximately 5,000 species described worldwide; however, as many as 20,000 species are estimated to exist[4]. Chironomids are useful in documenting water and habitat quality in many aquatic ecosystems because of their high diversity and variable pollution tolerance levels[5]. Furthermore, they are often the most abundant and widespread benthic macroinvertebrates in aquatic systems, typically accounting for 50% or more of the species in the community[5,6]. Following emergence of the terrestrial adult, the pupal exuviae (cast pupal skin) remains floating on the water's surface (**Figure 1**). Pupal exuviae accumulate along banks or behind obstructions through the action of wind or water current and can be easily and rapidly collected to give a comprehensive sample of chironomid species that have emerged during the previous 24-48 hr[7].

The relative abundance and taxonomic composition of collected SFPE reflects water quality, considering that some species are very pollution tolerant, while others are quite sensitive[5]. The SFPE method has many advantages over traditional larval chironomid sampling techniques including: (1) minimal, if any, habitat disturbance occurs at a sampling area; (2) samples do not focus on collecting living organisms, but rather the non-living skin, so the trajectory of community dynamics is not affected; (3) identification to genus, and often species, is relatively easy given appropriate keys and descriptions[3]; (4) collecting, processing, and identifying samples is efficient and economical in comparison to

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traditional sampling methods[3,8,9]; (5) accumulated exuviae represent taxa that have originated from a wide range of microhabitats[10]; (6) the method is applicable in nearly all aquatic environments, including streams and rivers, estuaries, lakes, ponds, rock pools, and wetlands; and (7) SFPE maybe be a more sensitive indicator of ecosystem health since they represent individuals that have completed all immature stages and successfully emerged as adults[11].

The SFPE method is not a new approach for gathering information about chironomid communities. Use of SFPE was first suggested by Thienemann[12] in the early 1900s. A variety of studies have used SFPE for taxonomic surveys (*e.g.,* [13-15]), biodiversity and ecological studies (*e.g.* [7,16-19]), and biological assessments (*e.g.,* [20-22]). Additionally, some studies have addressed different aspects of sample design, sample size, and number of sample events required for achieving various detection levels of species or genera (*e.g.,* [8,9,23]). These studies indicate that relatively high percentages of species or genera can be detected with moderate effort or expense associated with sample processing. For example, Anderson and Ferrington[8] determined that based on a 100-count subsample, 1/3rd less time was required to pick SFPE samples compared to dip-net samples. Another study determined that 3-4 SFPE samples could be sorted and identified for every dip-net sample and that SFPE samples were more efficient than dip-net samples at detecting species as species richness increased[3]. For example, at sites with species richness values of 15-16 species, the average dip-net efficiency was 45.7%, while SFPE samples were 97.8% efficient[3].

Importantly, the SFPE method has been standardized in the European Union[24] (known as chironomid pupal exuviae technique (CPET)) and North America[25] for ecological assessment, but the method has not been described in detail. One application of the SFPE methodology was described by Ferrington, *et al.*[3]; however, the primary focus of that study was to evaluate the efficiency, efficacy, and economy of the SFPE method. The purpose of this work is to describe all steps of the SFPE method in detail, including sample collection, laboratory processing, slide mounting, and genus identification. The target audience includes graduate students, researchers, and professionals interested in expanding traditional water quality monitoring efforts into their studies.

Protocol

1. Preparation of Field Collection Supplies

1. Determine the number of SFPE samples that should be collected based on the study design and acquire one sample jar (*e.g.,* 60 ml) for each sample.
2. Prepare two date and locality labels for each sample jar. Place one on the inside and affix the other to the outside of the jar. Ensure that each date and locality label includes the following information: country, state, county, city, water body, GPS coordinates, date, and name of person(s) collecting the sample.
3. Gather other specific materials and equipment (see Table of Specific Materials/Equipment).

2. Field Collection

1. Hold a larval tray in one hand and a sieve in the other. Dip the larval tray into the water where SFPE accumulate (*e.g.,* foam accumulations, snags, emergent vegetation, debris, back eddies, and along bank edges) (**Figure 2A**), allow water, exuviae, and debris to enter the larval tray, and pour this material through the sieve. If sampling in a lotic system, begin at the downstream end of the sample reach and work upstream (**Figure 2B**). If sampling in a lentic system, begin at the downwind shoreline.
    1. Repeat step 2.1 for 10 min (or as otherwise defined for a specific sampling regime) within each pre-defined sample reach (typically 100-200 m for samples collected from streams, but dependent on the overall area of the aquatic monitoring site); move between SFPE accumulation areas as appropriate.
2. Concentrate debris in one area of the sieve using a squirt bottle filled with water from the sample site and carefully transfer SFPE sample to pre-labeled sample jar with the aid of forceps and a stream of ethanol from a squirt bottle. Fill sample jar with ethanol.
3. Repeat steps 2.1 to 2.2 for all samples.

3. Sample Picking

NOTE: The rest of this protocol pertains to a 300 SFPE subsample and may need to be modified for other subsample sizes. See Bouchard and Ferrington's[9] subsampling and sampling frequency guidelines for tailoring SFPE methods to meet study-specific goals and resources.

1. Allocate a 1-dram vial for each SFPE sample; prepare a date and locality label to place inside each vial and fill the vial ¾ full with ethanol.
2. Remove lid from the corresponding sample jar and check for attached pupal exuviae. Gently rinse contents off the lid onto a Petri dish using a squirt bottle filled with ethanol. Locate and remove label from the inside of the sample jar using forceps and gently rinse contents off the label onto the Petri dish. Set label aside.
3. Transfer the contents of the sample jar into a larval tray, rinsing with ethanol to ensure no SFPE remain in the sample jar. Transfer a portion of the pupal exuviae, residue, and ethanol from the tray to the Petri dish. Ensure that the sample is covered in ethanol.
4. Place the Petri dish under a stereo microscope. Systematically scan the contents of the Petri dish for pupal exuviae. Pick all pupal exuviae from the dish using forceps and place into the vial. Do not pick specimens that are broken (*i.e.,* do not have at least half of the cephalothorax and abdomen), dried, or compressed to avoid later identification problems.
   NOTE: Identification to species often requires that the entire specimen is present, though in some cases, genus-level identification may be possible with partial specimens.
    1. Swirl dish and scan for additional pupal exuviae, including any that could be stuck to sides of the dish, as well as, any small and translucent specimens that may not have be detected initially. Repeat until two consecutive scans reveal no additional pupal exuviae.

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5. Repeat steps 3.3 and 3.4 until all or 300 pupal exuviae have been picked. When 300 pupal exuviae have been picked, return the residue from the Petri dish to the larval tray and rinse the Petri dish with ethanol. Then, transfer the residue from the larval tray to the empty sample jar, add the date and locality label, and put the lid on the jar. Retain or dispose of residue according to project-specific protocols.

4. Sample Sorting

1. Pour all picked pupal exuviae from the labeled vial into a Petri dish filled with enough ethanol to just cover specimens.
2. Under a stereo microscope, separate specimens into distinct morphological groups (*i.e.,* morphotaxa) and place each morphotaxon into separately a labeled vials filled 3/4th full with ethanol.
    1. Utilize external morphological characteristics to separate chironomid morphotaxa. For example, from the cephalothorax, use differences in the presence, size, shape, and coloration of the cephalic tubercles, frontal warts, frontal setae, and thoracic horn. From the abdomen, use spines, hookrows, shagreen, setae, and spurs of the abdominal segments, in addition to the anal lobes for morphotaxa separation (**Figure 4A**). See Ferrington, *et al.* [5], Sæther [26], Pinder and Reiss [27] for additional descriptions and figures of morphological characteristics.
    2. Use additional ethanol if specimens begin to dry.

5. Slide Mounting

1. Fill one well of a multi-well plate for each morphotaxon with 95% ethanol.
    1. Place multiple representations (*e.g.,* 25% of total) of each morphotaxon to be slide mounted into individual wells of the plate. Allow specimens to sit in well for at least 10 min to dehydrate sufficiently.
2. Label slides with appropriate site, collection, and identification information (**Figure 3**).
3. Place slide on the stereo microscope.
   NOTE: A template of the slide taped to the stage is useful for consistent placement.
4. Place a drop of Euparal on the slide; spread the Euparal so that it approximates the size of the coverslip. Use proper ventilation when working with Euparal.
   NOTE: Use proper ventilation when working with Euparal.
5. Embed a representative from the first morphotaxon into the Euparal using forceps.
   NOTE: To void excess ethanol from the specimen, using a forceps, gently tap specimen on laboratory wipes prior to embedding it in Euparal.
6. Separate the cephalothorax from the abdomen using fine-tipped forceps and/or dissection probes (**Figure 4A**).
    1. Split the cephalothorax along the ecdysial suture (**Figure 4B**) and open the cephalothorax so that the suture edges are on opposite sides (**Figure 4C**).
    2. Orient the cephalothorax so that the ventral side is facing up (**Figure 4C**).
    3. Position the abdomen dorsal side up; place immediately below the cephalothorax (**Figure 4C**).
7. Place a coverslip on the specimen. Hold coverslip at an angle, with one edge touching the slide, and then slowly lower and drop the coverslip to reduce air bubble formation. Press lightly on the coverslip to flatten the specimen.
8. Repeat steps 5.3 through 5.7 for all dehydrated specimens.

6. Genus Identification

1. Determine genus of slide-mounted specimens using a compound microscope. Identify specimens to genus using keys and diagnoses in Wiederholm 28 and Ferrington, *et al.*[5]. If needed, confirm family-level identification using Ferrington, *et al.*[5]. NOTE: There have been numerous generic descriptions and revisions since Wiederholm[28] and Ferrington, *et al.*[5], therefore, these keys and diagnoses are incomplete and need to be supplemented with primary literature.

Representative Results

Figure 1 illustrates the chironomid life cycle; immature stages (egg, larva, pupa) typically take place in, or closely associated with, an aquatic environment. Upon completion of the larval life stage, the larva constructs a tube-like shelter and attaches itself with silken secretions to the surrounding substrate and pupation occurs. Once the developing adult has matured, the pupa frees itself and swims to the surface of the water where the adult can emerge from the pupal exuviae. The exuviae fills with air, and by virtue of an outer waxy layer of the cuticle, it remains floating on the water surface until bacteria begin to decompose the wax layer.

Water currents or wind concentrate floating pupal exuviae into areas of accumulation, such as where riparian vegetation or fallen trees make contact with the water surface, illustrated in **Figure 2A**. A larval tray and sieve can be used to collect pupal from these natural accumulation areas and evaluate the emergence of Chironomidae from a broad spectrum of microhabitats, as shown in **Figure 2B**. For certain applications, it is important to collect samples in a consistent, standardized manner so that comparisons can be made among several sample sites or over time at a given sample site. Ten-minute collection periods have been shown to provide adequate evaluations of chironomid relative abundance[3,25]. For example, Ferrington, *et al.*[3] examined emergence estimates of the species *Chironomus riparius* and found that estimates did not vary substantially after 12 pan dips were analyzed. Within a 10-min collection period, many more than 12 dips are typically obtained, thus we feel confident that the majority of abundant species within a sample reach will be detected in this timeframe.[3]

Once SFPE samples have been collected, picked, and sorted, specimens are slide mounted for genus or species identification and creation of voucher specimens. Labeling the slides with appropriate site, collection, and identification information is recommended, as in **Figure 3**. Typically, the locality label displays information about the country, state, water body, GPS coordinates, study site ID, collection date, and

the name of person that collected the sample. Additionally, this label will have a unique slide number for each slide-mounted specimen. The identification label shows the genus and species (when applicable) identification and name of the person that identified the specimen.

Pupal exuviae need to be correctly dissected and oriented for genus identification and voucher specimen preparation. **Figure 4A** shows the correct dorsal side up pupal exuviae placement on the slide. During placement onto the slide, specimens may not initially lie dorsal side up because they are cylindrical in shape and often filled with ethanol and air bubbles. Therefore, using forceps or a dissection probe to slightly compress the abdomen into the Euparal towards the slide is suggested. Compression should orient the specimen in dorsal view and expel most of the ethanol and air bubbles. **Figure 4B** demonstrates the dissection that separates the cephalothorax from the abdomen. During this dissection, it is typical for beginners to tear the abdomen between the first and second abdominal segment. Caution should be placed in maintaining the first abdominal segment with the rest of the abdomen. **Figure 4C** shows the correct dissection and orientation of the pupal exuviae before positioning of the coverslip. For some specimens, it can be difficult to open the cephalothorax so that the suture edges are on opposite sides and the cephalothorax is oriented in ventral view. Again, a slight dorsoventral compression of the cephalothorax to achieve this placement is recommended.

Collections of SFPE have been successfully used in urban lakes in Minnesota to determine accumulation of species (**Figure 5A**) and genus richness (**Figure 5B**) and cumulative species composition along a gradient of mean phosphorus concentration/mean lake depth (**Figure 6**)[23]. Based on these results, a proof-of-concept study has been implemented for long-term monitoring of Chironomidae in relation to climate change in sentinel lakes across Minnesota (http://midge.cfans.umn.edu/research/biodiversity/chironomidae-slice-lakes/). Rufer and Ferrington[23] determined that four SFPE samples per lake per season recovered the majority of the chironomid community and detected important seasonal variation in urban lakes (**Figure 5A, B**). In all 16 lakes, April samples contained different taxa than May through September samples. Therefore, in northern-temperate regions, sampling four times per season is recommended, with one sample in April and three samples between May and September. However, for different geographic areas and climates, the sampling regime should be tailored to the region to maximize the portion of the community collected.

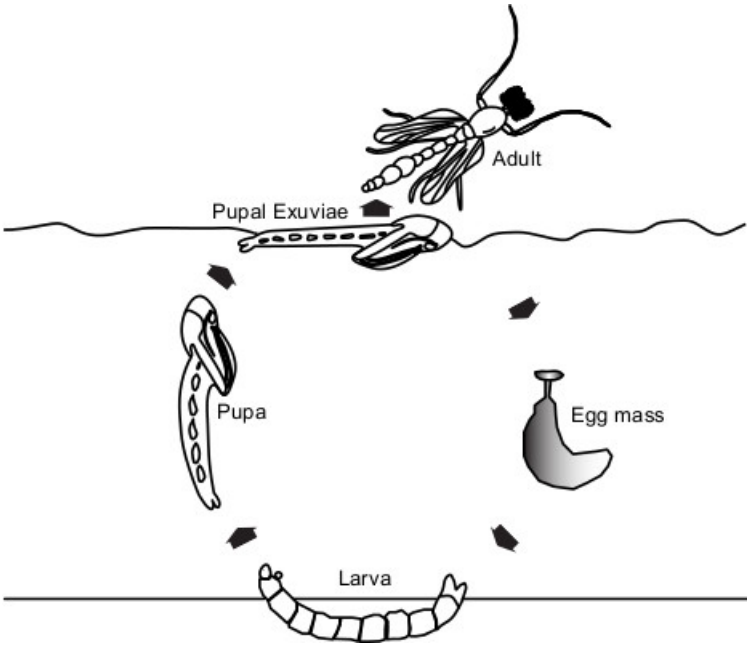

Figure 1. Chironomid life cycle. There are four life stages, egg, larva, pupa, and adult, in the chironomid life cycle. Female adults lay eggs on the surface of the water. Eggs sink to the bottom and typically hatch in several days to one week. After leaving the egg mass, larvae burrow into the mud or construct small tubes in which they live, feed, and develop. Larvae transform into pupae while still in their tubes. After pupation, pupae actively swim to the surface of the water and adults emerge from the pupal exuviae. Please click here to view a larger version of this figure.

Figure 2. Examples of an area of SFPE accumulation and field collection techniques in a stream. (**A**) An example of where SFPE would accumulate upstream of a log. The white, foamy material is a combination of organic matter, such as macrophytes and algae, and can contain hundreds to thousands of pupal exuviae. (**B**) An example of how a collector would use a sieve and larval tray to collect SFPE from the riparian banks of the stream. Please click here to view a larger version of this figure.

Figure 3. Diagram showing locations of slide date and locality label (left), identification label (right), and slide mounted pupal exuviae under coverslip (center). Please click here to view a larger version of this figure.

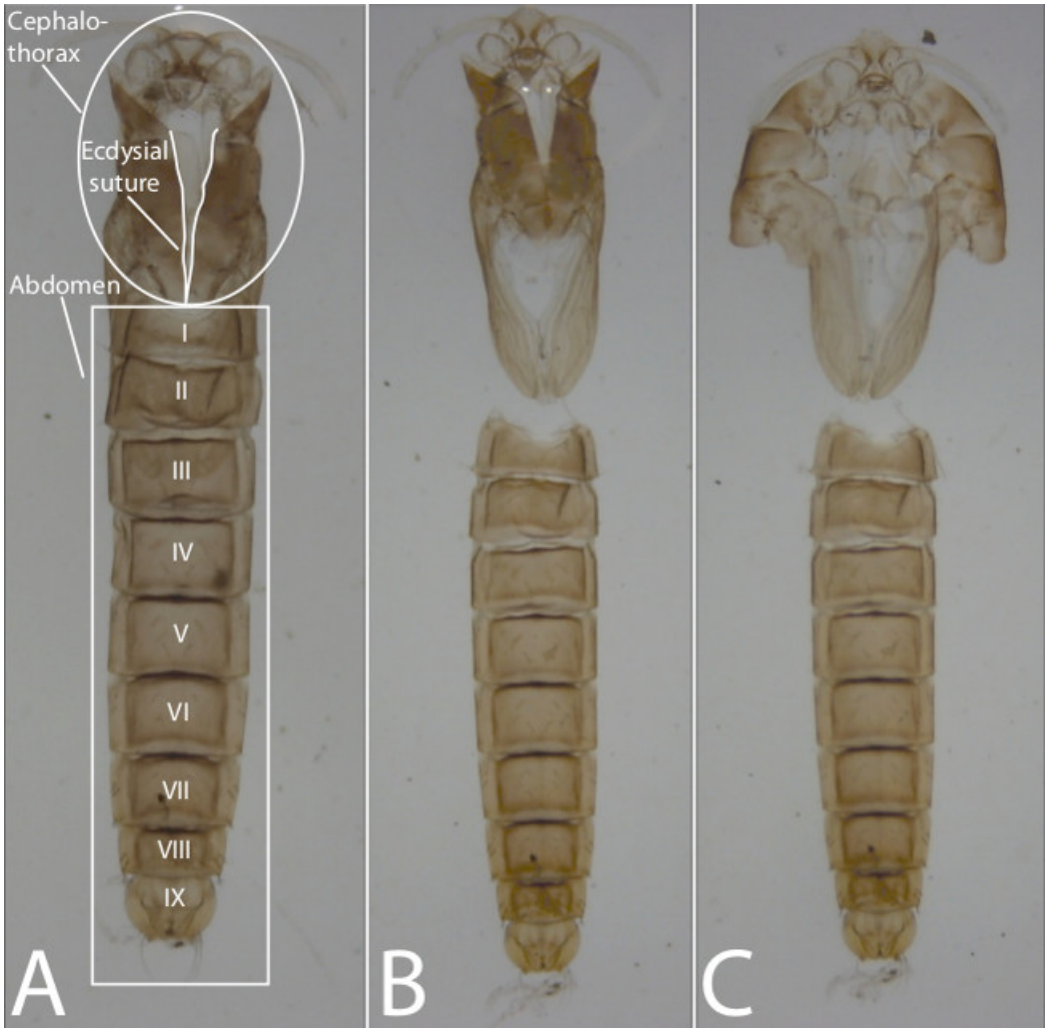

Figure 4. Step-by-step pupal exuviae dissection and orientation. (**A**) Undissected pupal exuviae (cephalothorax and abdomen with segments numbered in dorsal view). (**B**) Dissected pupal exuviae (cephalothorax and abdomen in dorsal view). (**C**) Dissected and oriented pupal exuviae (cephalothorax: ventral view; abdomen: dorsal view). Please click here to view a larger version of this figure.

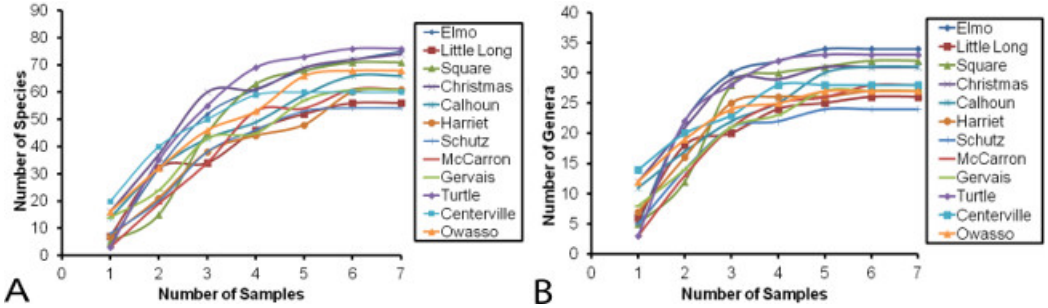

Figure 5: Taxonomic accumulation curves for SFPE samples collected from 16 urban lakes in Minnesota. For both panels, each colored line represents one of the 16 lakes. See Rufer and Ferrington [23] for a detailed description of the characteristics of each lake. Each data point represents a monthly 10-min SFPE sample collected along the downwind shore during the ice-free months of 2005 (April to October). A) Species accumulation curves for SFPE samples. B) Genus accumulation curves for SFPE samples. Please click here to view a larger version of this figure.

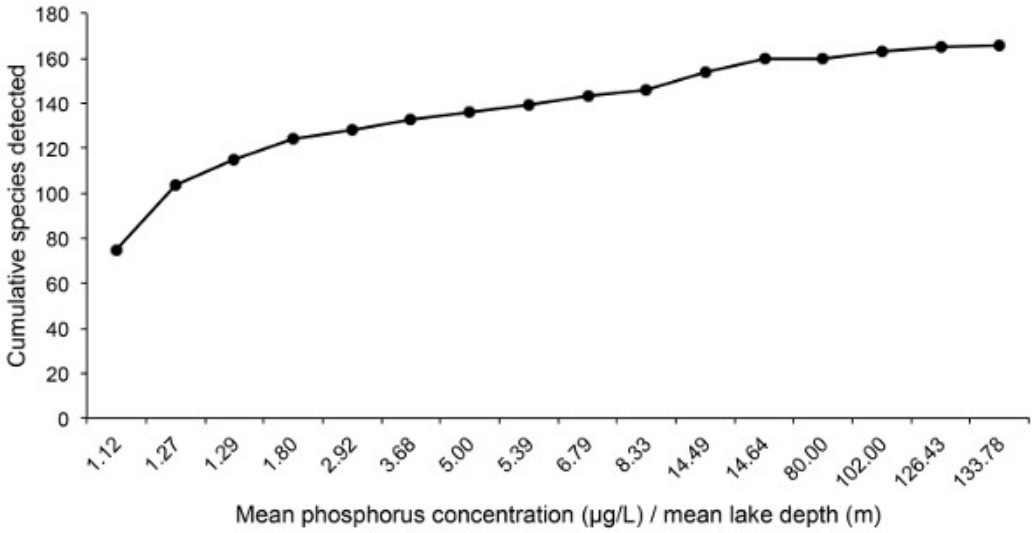

Figure 6: Cumulative species detected across a gradient of lake chemistries from multiple SFPE samples as a function of mean epilimnetic phosphorus concentration (µg/L) over mean lake depth (m) from 16 urban lakes in Minnesota. Each data point represents one of the 16 lakes; lakes are sorted from lowest to highest mean phosphorus/mean depth. See Rufer and Ferrington 23 for a detailed description of the characteristics of each lake. Cumulative number of species encountered increases as the ratio of mean phosphorus concentration over mean lake depth increases.

Discussion

The most critical steps for successful SFPE sample collection, picking, sorting, slide mounting, and identification are: (1) locating areas of high SFPE accumulation within the study area during field collection (**Figure 2A**); (2) slowly scanning the contents of the Petri dish for detection of all SFPE during sample picking; (3) developing the necessary manual dexterity to dissect the cephalothorax from the abdomen during slide mounting (**Figure 4A**); and (4) recognizing key morphological characters of chironomid pupal exuviae to correctly identify to genus.

Detecting areas of high SFPE accumulation (**Figure 2A)** is the most important step in successful SFPE sample collection. Pupal exuviae are caught in aquatic vegetation or human structures like boat ramps, and waves can concentrate floating material into offshore "windrows"[30]. For larger bodies of water, the identification of natural areas of accumulation may require locating study sites based on wind patterns or using watercraft to access areas where pupal exuviae are amassing. A sample with a sufficient number of SFPE needs to be collected to detect the presence of emerging species and estimate the relative abundance of individual species with a high degree of accuracy. During sample sorting, it is necessary to slowly scan the Petri dish multiple times for smaller (3-6 mm in length), lightly pigmented specimens. SFPE often stick to algae, leaves, sticks, seeds, and flowers, and therefore, may not be detected during the initial scan. Also, this protocol requires careful dissection and slide mounting of the cephalothorax from the abdomen for genus identifications (**Figure 4A)**. Use fine-tipped forceps and/or dissection probes to dissect exuviae between the cephalothorax and first abdominal segment. Finally, genus identification can be difficult for new taxonomists. Take the time to study morphology and terminology of chironomid pupae before starting to identify specimens to genus. See Wiederholm [28] and Ferrington, *et al.* [5] for keys and diagnoses of chironomid genera. If identification skills are a concern, all slides or a subset of voucher specimens can be sent to a laboratory with the appropriate abilities.

Based on staggered adult emergences in most communities, multiple sampling events are advised, and for long-term studies, a pilot project can determine the most useful sampling times prior to finalizing methods. Even with multiple, seasonally targeted sampling events, a proportion of the community will remain undetected, although these are often rare taxa[31]. For sampling frequency recommendations, see Bouchard and Ferrington [9] for streams and Rufer and Ferrington [23] for lakes. The main concern regarding sampling methodology relates to SFPE floating distance. In streams, typical drift is between 50-250 m, whereas in larger rivers exuviae may move up to 2 km[30]. Field evidence suggests that fifty percent or more of the exuviae do not displace more than 100 meters downstream of where the adult emerges[20]. Therefore, if one is collecting SFPE over a sample reach of 500 meters downstream from a suspected pollution source, it is likely that the majority of the specimens collected completed their life cycle within the suspected impact zone[25]. In lakes, ponds, and pools, pupal exuviae will move with surface currents and often collect in large numbers on the downwind side of the water body.

Although cost-efficient, there are potential limitations associated with this method, including: (1) the inability to determine microhabitats used by larvae[32]; (2) the inability to assess major lifecycle events and instar duration prior to eclosion, since voltinism is often challenging to determine[7]; (3) strong seasonal variability to assemblages detected[30]; (4) a bias against species with lightly chitinized exuviae that break down or sink at a faster rate[33]; (5) not being able to identify specimens to species if pupae and adult males have not previously been associated[5]; and (6) the difficulty of estimating areal density or biomass.

As described above, pupal exuviae are among the most useful and cost-efficient life stages to include in aquatic biomonitoring studies[5]. Future studies to improve the SFPE method include testing: (1) appropriate replications; (2) subsample sizes; (3) appropriate frequency of sampling events depending on locality and water body of interest; and (4) sinking and breakdown rates for exuviae under various conditions of

temperature, humidity, decomposer inoculation, and mechanical disturbances. In addition, future studies should include refinement of molecular-based identification techniques, such as DNA barcoding, to associate pupal exuviae with larvae and adults[34-35].

Here we have described chironomid SFPE sample collection, laboratory processing, slide mounting, and genus identification in detail. The SFPE method is efficient for assessing diverse, widespread chironomid communities and can augment benthic samples in studies of biological responses to changing water quality. This cost-effective, alternative RBP offers several distinct advantages that make it well-suited for large-scale analyses that include repeated sampling events over extended periods of time.

Disclosures

The authors declare that they have no competing financial interests.

Acknowledgements

Funding for composing and publishing this paper was provided through multiple grants and contracts to the Chironomidae Research Group (L. C. Ferrington, Jr., PI) in the Department of Entomology at the University of Minnesota. Thanks to Nathan Roberts for sharing fieldwork photographs used as figures in the video associated with this manuscript.

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