Cumulative Effects of Coastal Watershed Land Use on Chironomidae (Insecta: Diptera) Communities of Neotropical Estuaries in Costa Rica

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Dedication

This dissertation is dedicated to my parents, Lynne and Ronald Tchida, and my favorite field assistants.

Abstract

Land use change and intensification significantly impact estuarine and coastal ecosystems. On the Caribbean coast of Costa Rica, sediments and nutrients transported from watersheds converted from forested to agricultural or urban landscapes have consequences on these productive ecosystems. Chironomidae assemblages are likely to provide a useful measure of biotic integrity in Neotropical estuaries of Costa Rica, which lack an intensive estuarine bioassessment tool to support environmental monitoring. However, little is known about chironomid communities in these estuaries and the cumulative effects of watershed land use on chironomid communities have not been studied in these estuaries. The purpose of this dissertation was to (1) quantify land cover change of six watersheds on the Caribbean coast of Costa Rica between 2001 and 2014, (2) describe all steps of the Chironomidae surface-floating pupal exuviae method in detail, including sample collection, laboratory processing, slide mounting, and genus identification, (3) investigate Chironomidae species diversity of nine estuaries across a land use gradient on the Caribbean coast of Costa Rica, (4) compare the relative effectiveness of five different DNA extraction protocols and direct PCR in isolation of DNA from chironomid pupal exuviae, and (5) assess the efficiency of using standard DNA barcoding for species identification of chironomid pupal exuviae. Watershed-scale land use analyses showed agricultural expansion and deforestation in watersheds on the northeastern coast and secondary forest regrowth on the southeastern coast of Costa Rica. Chironomids are valuable bioindicators of water quality, since some genera and some species are more tolerant to pollution than others. Specifically, relative abundance and

species composition of Chironomidae surface-floating pupal exuviae samples reflect changes in water quality. I identified 228 morphospecies and 70 genera from 17,071 Chironomidae surface-floating pupal exuviae collected from nine Neotropical estuaries and a Chironomidae Index of Biotic Integrity successfully discriminated estuaries with differing degrees of stress across a land use gradient. Future biodiversity studies and water quality assessments should target their research efforts on watersheds that are most ecologically damaged and at risk, like Tortuguero. Genomic DNA was extracted from 61.2% of 570 sampled pupal exuviae. The NucleoSpin® Tissue XS Kit, DNeasy® Blood and Tissue kit, and QuickExtract™ DNA Extraction Solution provided the best results in isolating DNA from single pupal exuviae. A total of 69 out of 190 (36.3%) chironomid pupal exuviae resulted in high-quality sequences for Costa Rica, but none matched known species. I found effective protocols for isolating DNA from chironomid pupal exuviae; however, my results indicate that association of unknown specimens to named species suffers from the incompleteness of the barcode reference library for Chironomidae from this region. This dissertation is the first large-scale study to conduct concurrent geospatial and biological monitoring of multiple estuaries on the Caribbean coast of any country in Central America. This knowledge is important if these aquatic communities are to be used more effectively in future biological monitoring, conservation, and integrated water resource management of Neotropical estuaries.

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CHAPTER 1: Land use and land cover change in six watersheds on the Caribbean coast of Costa Rica with implications for estuarine monitoring

Summary

Estuarine and coastal ecosystems on the Caribbean coast of Costa Rica contribute to important ecosystem services including biogeochemical cycling and maintenance of biodiversity. However, changing land use, from native rainforest to monoculture agriculture or urban, has posed significant risks to these ecosystems. As agricultural production increases, watershed-scale analyses are crucial for relating land use changes to water quality. I quantified land cover change of six watersheds on the Caribbean coast of Costa Rica between 2001 and 2014. The overall five-class classification accuracies for all six watersheds averaged 84% for 2001 and 79% for 2014. Over the 13-year period, in the Tortuguero watershed on the northern coast, there was a reduction in forest (57% to 54%) and a gain in agriculture (8% to 10%) and urban (6% to 7%) land cover, which has potential to result in declining water quality in the estuary. By contrast, in Estrella watershed on the southern coast, there was a gain in forest (87% to 91%), no change in agriculture (3% to 3%), and a reduction in pasture (8% to 4%) land cover, with potential to improve estuarine water quality. My results show agricultural expansion and deforestation in watersheds on the northern coast and secondary forest regrowth on the southern coast that can be linked to biological responses related to water quality trends. The results of this study can serve as a model for linking conservation and management programs at large spatial scales in tropical watersheds and predicting long-term water

quality trends within estuarine and coastal ecosystems.

Introduction

Estuaries and coastal ecosystems (ECEs) comprise some of the world's most productive habitats (Bierman et al. 2011) and provide valuable ecosystem goods and services, such as raw materials and food, maintenance of biodiversity, tourism, nutrient cycling, erosion control, water purification, and carbon sequestration (Barbier et al. 2011). Despite their ecological and societal importance, ECEs are at risk to many human-induced pressures, including sedimentation, eutrophication, and chemical pollution, as terrestrial ecosystems are converted from forested lands to agricultural or urban areas (Wolanski and Elliott 2016). Therefore, it is necessary to monitor these coastal environments at large spatial scales, since land use-related changes in water quality can have strong implications on ECEs goods and services that benefit human beings and biodiversity (Barbier et al. 2011).

The importance of large-spatial scale assessments was recognized by Wolanski et al. (2004), who suggested that to maintain healthy, productive ECEs, land use changes in the entire river catchment need to be considered, including the headwaters through the catchment and estuary and down to the coastal zone. Landscape patterns can play an important role in water quality variation (Basnyat et al. 1999; Huang and Klemas 2012; Huang et al. 2011) through nonpoint sources of pollution from land runoff (Zhou et al. 2014). As a result, understanding the causes of water pollution needs an integrative effort at the watershed scale (Carey et al. 2011; Kearns et al. 2005). Therefore, quantification of

land-use and land-cover (LULC) change is crucial for integrated coastal watershed assessment and predicting long-term water quality trends in ECEs.

Remote sensing has been used to effectively analyze LULC changes at the coastal watershed scale (Huang and Klemas 2012; Zhou et al. 2014) in North America (Berlanga-Robles and Ruiz-Luna 2011; Nosakhare et al. 2011; Wang et al. 2014; Wimberly and Ohmann 2004; Xian and Crane 2005), South America (Armesto et al. 2010; Bertolo et al. 2012; Maeda et al. 2008), and Europe (Alphan and Yilmaz 2005; Esen and Uslu 2008; Symeonakis et al. 2007; Teixeira et al. 2014). However, in Costa Rica, only a few LULC change studies have been completed in coastal watersheds on the Pacific coast (Algeet-Abarquero et al. 2014; Corcoran et al. 2014; Daniels and Cumming 2008; Sanchez-Azofeifa et al. 2002). One study focused on the Caribbean coast (Vallet et al. 2016); however, this study analyzed the implications of forest cover change on ecosystems services over time, such as water regulation, in a single Costa Rican watershed, Reventazón (Vallet et al. 2016).

The Caribbean coast of Costa Rica presents some of the world's greatest challenges and opportunities for coastal watershed conservation and management (Fraixedas et al. 2014; Grant et al. 2013; Guzmán and Jiménez 1992; Neeman et al. 2015; Sánchez-Azofeifa et al. 2007; Wehrtmann and Cortes 2009). Costa Rica is one of the world's richest hotspots of biodiversity (Myers et al. 2000) with the Caribbean provinces (Cartago, Heredia, and Limón) having the highest density of extinct, endangered and threatened species of mammals, birds, amphibians and reptiles in Costa Rica (Jadin et al. 2016). It has been recognized internationally as a leading country in natural habitat

protection by managing over 26% of its terrestrial land as protected areas (The World Bank 2015). The Caribbean coast of Costa Rica has four protected areas: Barra del Colorado Wildlife Refuge (789.8 km^2), Tortuguero National Park (261.6 km^2), Cahuita National Park (10.7 km^2), and Gandoca-Manzanillo Wildlife Refuge (50.1 km^2) (SINAC 2016). Since the 1980s, management of these protected areas has led to reforestation and spontaneous regrowth, and forest area is now considered stabilized or increasing in some parts of the Caribbean coast of Costa Rica (Vallet et al. 2016).

Over the same time period, Costa Rica has become a leader in the export of tropical fruit (FAO 2016) and has achieved some of the highest yields per hectare of bananas and pineapples in the world (Fagan et al. 2013). The area of pineapple harvested increased by over 353% from 13,035 to 46,000 hectares between 2001 and 2013, respectively (FAO 2016). In particular, the Caribbean coast of Costa Rica is a prime banana and pineapple-producing area as a result of warm temperatures, high rainfall, and soils with extensive drainage systems where surplus water flows into streams and rivers (Grant et al. 2013). However, the intensive production of banana and pineapple leads to the use of large quantities of fertilizers and pesticides (Castillo et al. 2006; Castillo et al. 2000; Quijandría et al. 1997) and increases in sedimentation as a result of runoff from deforested riparian zones that can contaminate downstream ECEs (Vargas Ramírez 2007) and harm the taxa that they support (Henriques et al. 1997). Grant et al. (2013) found that pesticides prompted toxic effects in spectacled caiman (*Caiman crocodilus*) captured in Tortuguero Conservation Area, one of Costa Rica's most important wilderness areas, as a

result of diminished overall health or the quantity or quality of prey was reduced by pesticides downstream of banana plantations.

The purpose of this study is to evaluate land cover composition across and within six watersheds on the Caribbean coast of Costa Rica during a 13-year period as part of a long-term coastal watershed water quality assessment program. The present study is part of a larger study designed to link coastal watershed LULC changes and estuarine quality parameters, with a goal of quantifying and predicting the impacts of land use on estuarine water quality and aquatic biodiversity of the Caribbean coast of Costa Rica.

Materials and Methods

Study area

The study area includes six watersheds (Chirripo, Tortuguero, Reventazón, Pacuare, Bananito, Estrella) on the Caribbean coast of Costa Rica with a total area of approximately 9,982 km^2 and located roughly between 9°00' and 11°00' N latitude and 83°00' and 84°00' W longitude. These six watersheds were selected to represent a land use gradient from mostly primary and secondary tropical rainforest to largely covered with monoculture plantation agriculture (e.g. banana and/or pineapple). In addition, they drain into nine estuaries studied as part of a larger estuarine bioassessment study that did not include estuaries within the Limón watershed. Elevation in the study area ranges from sea level to 3,461 m in the Central Cordilleras (i.e., mountain ranges) (Fig. 1.1). These steep topographic gradients cause the land cover types to change rapidly over short distances. The study watersheds include three geological units: (1) the Caribbean

lowlands which are filled with alluvial and marine sediments, (2) the young Central Cordillera which is composed of active volcanoes, and (3) the Talamanca Cordillera which is dominated by old sedimentary and volcanic rocks (Nieuwenhuyse 1996). The climate of the Caribbean coast of Costa Rica consists of two rainy seasons, one from November to March and a second from June to August. Annual rainfall rate in the northern Caribbean coast is approximately 6 m and in southern coast is approximately 2.5 m (Cortés et al. 2010). Average annual air temperatures are 25.9°C (IMN 2016).

Image pre-processing

For 2001 era maps, I selected Landsat imagery with less than ≤1% cloud cover, plus four spectral bands (Landsat bands 2-5) and calculated three vegetation indices from the Landsat data (Table 1.1). The vegetation indices were Normalized Difference Vegetation Index (NDVI), Normalized Differences Water Index (NDWI,) and a normalized difference of Band 2 and Band 5 that distinguished banana cultivation (ND25, calculated like NDVI). The NDVI and NDWI were computed for each pixel using the following equations:

$$NDVI = \frac{\rho(\text{band } 4) - \rho(\text{band } 3)}{\rho(\text{band } 4) + \rho(\text{band } 3)},$$

$$ND25 = \frac{\rho(\text{band } 2) - \rho(\text{band } 5)}{\rho(\text{band } 2) + \rho(\text{band } 5)},$$

and

$$NDWI = \frac{\rho(\text{band } 4) - \rho(\text{band } 5)}{\rho(\text{band } 4) + \rho(\text{band } 5)}.$$

For 2014 era maps, I was unable to find suitable Landsat imagery with <20% cloud cover for the study area. Therefore, I selected circa year 2014 Landsat 8 cloud-free image composites that covered the study area (two 10x10 degree granules at 10N/90W and 20N/90W) (Hansen et al. 2013). I included the bands that were provided by Hansen et al. (2013) (median reflectance values of multitemporal Landsat bands 3-5, 7) plus derived NDVI and NDWI. I did not include derived ND25 since Landsat band 2 was not available in the composite images.

I mosaicked and clipped satellite images by elevation thresholds within watershed boundaries. Specifically, I used a void-filled version of the Shuttle Radar Topographic Mission (SRTM) digital elevation model (DEM) (v. 2.1) at 90-meter resolution to derive elevation and the SRTM-derived drainage basins data set to produce watershed boundaries (Farr et al. 2007). Then, I separated images into three elevation thresholds (0 to 50 m, 51 to 500 m, and 501 and above m) to avoid confusion between shadows and rock outcroppings with dark vegetation and water features.

Image classification and land cover change detection

I used ERDAS Imagine version 2013 to run ISODATA unsupervised classifications with 20-30 initial classes. These initial classes were later merged into seven land cover classes: forest, row crop, pasture, urban, water, cloud, and other. Detailed descriptions of these land cover classes are given in Table 1.2. Next, I performed additional ISODATA unsupervised classifications (also known as "cluster busting") for each land cover class with 10-20 initial classes that were later merged into

the final seven land cover classes. This technique was used to reduce class confusion and improve classification results (Corcoran et al. 2014; Ozesmi and Bauer 2014).

The classified images were filtered to reduce speckle noise and smooth the distribution of land cover classes. A 3x3 majority filter was used to recalculate values using the nearest neighbor approach. Change detection matrices were calculated for each land cover class using a post-classification comparison method. The post-classification method compares individual classification on a pixel-to-pixel basis to extract "from-to" change information (Jensen 2005).

Classification accuracy assessment

An independent stratified random sample method was used to create 75 reference points for each land cover class for a total of 375 points for each watershed by year map. I interpreted each sample point using high-resolution aerial orthophotos and satellite images as the reference data. For the 2001 era maps, I used 0.50-meter resolution 2003 CARTA aerial photos, high-resolution satellite imagery accessed via DigitalGlobe 2016, and 30-meter resolution Landsat imagery. For the 2014 era maps, I used high-resolution satellite imagery for 2010 to 2015 accessed via DigitalGlobe 2016 and Google Earth 2016. I evaluated the land cover maps using a single pixel based approach based on the analysis of error matrix (Congalton and Green 2009) found in the software package in the RS Accuracy v. 0.96 (Knight 2001). The following accuracy assessment estimators were computed: error matrices, overall accuracy, producer's accuracy, user's accuracy, and kappa statistics (K-hat).

Results

Classification accuracy assessment

The average overall accuracy for 2001 was 84% with a Kappa statistic of 0.80, where the Tortuguero watershed had the highest overall accuracy at 90% with a Kappa statistic of 0.88. For 2014, the average overall accuracy was 79% with a Kappa statistic of 0.74 and the Pacuare watershed map had the highest overall accuracy at 85% with a Kappa statistic of 0.81. User's and producer's accuracies for individual land cover classes ranged from 52% to 100% and from 47% to 100%, respectively. In the 2001 era classification, the average accuracy (combined user's and producer's) of each land cover class was the following (listed in decreasing order): water (91%), row crop (87%), forest (87%), urban (80%), and pasture (78%). In the 2014 era classification, the average accuracy (combined user's and producer's) of each land cover class was the following (listed in decreasing order): water (88%), forest (82%), row crop (80%), urban (79%), and pasture (76%) (Appendix A & B).

Land cover change analysis

Land cover dynamics during the study period (2001-2014) showed a general increase in row crop and urban areas and decrease in forest and pasture areas across watersheds on the Caribbean coast of Costa Rica (Fig. 1.2). In 2001 and 2014, Tortuguero watershed had the lowest total area of forest cover (57% and 54%, respectively), the highest total area of row crop cover (8% and 10%, respectively), and the highest total area of pasture cover (28% and 28%, respectively). In 2001 and 2014,

Pacuare watershed had moderate forest cover (79 and 76%, respectively), moderate row crop cover (7% to 6%, respectively), and moderate pasture cover (10% to 12%, respectively). In 2001 and 2014, the southern Estrella watershed, had the highest total area of forest cover (86% and 91%, respectively), the lowest total area of row crop (3% and 3%, respectively), and the lowest total area of pasture (8% and 4%, respectively) (Tables 1.3-1.4 & Fig. 1.4).

Over the 13-year period (2001-2014) across all six watersheds, there was an increase in row crop area (24%) and urban area (26%), while there was a decrease in water area (48%), pasture area (12%), and forest area (1%) (Table 1.5). The two most northern watersheds, Chirripo and Tortuguero, resulted in row crop area with the largest relative percent change. The largest relative percent change for the two most central watersheds, Reventazón and Pacuare, was urban area and for the two most southern watersheds, Bananito and Estrella, it was pastoral area (Table 1.5 & Fig. 1.3). In the Chirripo watershed, row crop area increased relatively by 106% from 92.6 km^2 (2% of the total area) to 190.5 km^2 (4% of the total area). In the Tortuguero watershed, water area decreased relatively by 49% from 15.8 km^2 (1% of the total area) to 8.0 km^2 (1% of the total area) and row crop area increased relatively by 28% from 102.5 km^2 (8% of the total area) to 130.7 km^2 (10% of the total area). In the Reventazón watershed, urban area increased relatively by 35% from 270.8 km^2 (11% of the total area) to 365.9 km^2 (15% of the total area). In the Pacuare watershed, urban area increased relatively by 78% from 24.7 km^2 (3.4% of the total area) to 44.1 km^2 (6% of the total area). In the Bananito watershed, water area decreased relatively by 65% from 2.1 km^2 (1% of the total area) to

0.7 km^2 (1% of the total area) and pasture area decreased relatively by 46% from 16.0 km^2 (8% of the total area) to 8.7 km^2 (5% of the total area). In the Estrella watershed, pasture area decreased relatively by 52.4% from 76.3 km^2 (8% of the total area) to 36.3 km^2 (4% of the total area).

Five major land-cover conversions occurred from 2001 to 2014: (1) old to young forests converted to row crop area (Chirripo and Tortuguero watersheds); (2) old to young forests converted to urban areas (Reventazón and Pacuare watersheds); (3) pastures converted to row crop area (Chirripo and Tortuguero watersheds); (4) pastures converted to urban areas (Reventazón watershed); and (5) pastures converted to young forests (Bananito and Estrella watersheds) (Table 1.6). In the northernmost Chirripo watershed, I found that a gain in row crop area was primarily due to a conversion of 62.8 km^2 of pasture and the conversion of 56.7 km^2 forest area to row crop (Table 1.6a). In the Tortuguero watershed, the gain in row crop area was due to a conversion of 31.7 km^2 of pasture and the conversion of 23.4 km^2 of forest to row crop. In addition, there was a loss of water area from a conversion of 8.9 km^2 of water area to forest (Table 1.6b). In the Reventazón watershed, the gain in urban area was due to a conversion of 94.1 km^2 pasture and the conversion of 87.2 km^2 of forest to urban (Table 1.6c). In the Pacuare watershed, the gain in urban area resulted mostly from a conversion of 15.9 km^2 forest area to urban (Table 1.6d). In the Bananito watershed, the loss in pasture area resulted mostly from a conversion of 10.6 km^2 of pasture to forest area. In addition, there was a loss of water area from a conversion of 1.3 km^2 of water area to forest (Table 1.6e). In

southernmost Estrella watershed, the loss in pasture area resulted mostly from a conversion of 47.2 km^2 pasture to forest area (Table 1.6f).

Discussion

Results from my study show five distinct patterns of land use change, from most to least likely to impact water quality conditions of the watershed: (1) Cropland expansion on tropical forests; (2) Urbanization on tropical forests; (3) Cropland expansion on pastures; (4) Urbanization of pastures; and (5) Forest recovery of abandoned pastures. These findings indicate distinct landscape changes across the study area on the Caribbean coast of Costa Rica, similar to those in other Costa Rican remote sensing studies (Algeet-Abarquero et al. 2014; Broadbent et al. 2012; Fagan et al. 2013; Lambin and Meyfroidt 2011; Zahawi et al. 2015). Since the mid-1990s, most of Costa Rica has experienced a reduction in deforestation rates due to effective Protected Areas (Redo et al. 2012) and Payments for Ecosystem Services (Robalino et al. 2015), stemming from a Forest Law passed in 1996, which introduced a permit system to restrict timber extraction and forest-cover change on private land (Arroyo-Mora et al. 2014; Calvo-Alvarado et al. 2009; Robalino and Pfaff 2013). While forest protection efforts have slowed mature forest loss, expansion of crops, like pineapple and bananas, are still causing deforestation of exotic and native tree plantations, wetlands, and secondary forests in the Caribbean lowlands of northern Costa Rica. This last decade, large-scale, highly profitable pineapple cultivations were introduced to the northeastern region of Costa Rica (Fagan et al. 2013). Pineapples can be grown in poor soils with good

drainage, and farms have expanded rapidly onto former pastures (Fagan 2014). I found this LULC change pattern in my northern most watersheds of Chirripo and Tortuguero with the area of row crop land cover increasing over time. In the Chirripo watershed, the row crop area doubled in size over the last 13 years. It appears that most of the crop expansion is occurring in the forested areas surrounding the perimeters of row crop fields, and as a result, the forest patches adjacent to these monoculture agricultural plantations have been cleared to make room for additional production. As can be seen in all my land cover classification results, row crops are often planted right next to water bodies, like streams and rivers, without forest cover in the riparian buffer zones. Forest cover adjacent to agricultural fields, especially when adjacent to water bodies, can mitigate the negative impacts of nutrient and pesticide leaching, spreading of pathogens, soil erosion, and reduction of ecosystems services provided by terrestrial and aquatic biodiversity (Gregory et al. 1991; Haddaway et al. 2016; Sweeney and Newbold 2014).

In addition to cropland expansion and tropical forest and pasture reduction, the Caribbean coast of Costa Rica has experienced urbanization with a concommitant increase in the number of paved and improved gravel roads to accommodate the northward movement of banana farming and the expansion of pineapple production (Fagan 2014). This urbanization of tropical forests and pastures is pronounced in the south-central watersheds of Reventazón and Pacuare. Costa Rica is deeply invested in improving the public infrastructure for pineapple and banana export. Currently, the Chinese government has funded the expansion of Route 32, which would improve road access to the capital, San José, from the province of Limón (Arias 2016). Also,

Netherlands-based APM Terminals is funding construction of Costa Rica's largest infrastructure project, the $1 billion Moín container terminal (Dyer 2015).

The most promising trend for improving water quality conditions over time can be seen in the forest recovery of the southeastern watersheds (i.e. Estrella and Bananito watersheds). Forest reserves and other forms of zoning of forestry land helped to control the rebound effect of agricultural intensification (Lambin and Meyfroidt 2011) since these lands were left to recover once secured in protected areas with strong incentives from the Payment for Ecosystem Services program (Calvo-Alvarado et al. 2009). Costa Rica experienced a net increase in reforestation of moist forest between 2001 and 2010 (Aide et al. 2013) and has had a substantial gain in moist forest biome near the borders with Panama and Nicaragua and in the central highlands (Redo et al. 2012). Lambin and Meyfroidt (2011) found that forests encroached mainly on abandoned or marginal land rather than prime agricultural land. My results suggest the same; abandoned or marginal pastures from either agriculture or cattle grazing were replaced by secondary forest regrowth in both Bananito and Estrella watersheds over the 13-year period.

My study had some technical limits due to errors in LULC classifications. Remote-sensing reflectance measurements cannot easily differentiate between some land uses. For example, areas of agroforests, like wild bananas, mixed with the agriculture land cover class; harvested pineapple plantations with bare soil mixed with the urban land cover class; and shaded ornamental plants and dark-colored cloud shadows mixed with the water land cover class. In addition, the moderate resolution of Landsat imagery (30 x 30 m) caused pixels that have a border with two land cover classes (e.g. water next to

agriculture) to get mixed. I recommend that future studies refine the LULC classes by using an Object-Based Image Analysis (OBIA) approach with high spectral resolution imagery, such as Worldview 3 or 4, high resolution elevation data, such as Lidar data, and contextual information to have a better interpretation of the classes for each watershed (Blaschke 2010; Rampi et al. 2014).

Based on my results, I would rank watersheds in the following order from most to least impacted by human land use change, and thus, susceptible to water quality issues: Tortuguero, Reventazón, Chirripo, Pacuare, Bananito, and Estrella. Future biodiversity studies and water quality assessments should target their efforts on watersheds that are most ecologically damaged, sensitive, and at risk, like Tortuguero. The results of this study will serve as a model for large-scale conservation and management of tropical watersheds and for predicting long-term water quality trends of tropical estuarine and coastal ecosystems.

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Tables and Figures

Table 1.1: Landsat image information.

Watershed(s)	Year	Acquisition date	Row/path	Type
Chirripo	2001	January 14, 2001	52/15	Landsat 5 TM
Chirripo, Tortuguero, Reventazón, Pacuare	2001	January 14, 2001	53/15	Landsat 5 TM
Bananito, Estrella	2001	December 12, 2001	53/14	Landsat 7 ETM+
Chirripo, Tortuguero, Reventazón, Pacuare, Bananito, Estrella	2014[1]			Landsat 8 OLI

[1]Reference multispectral imagery from the last available year, typically 2014. If no cloud-free observations were available for year 2014, imagery was taken from the closest year with cloud-free data, within the range 2010–2012.

Table 1.2: Land cover classification scheme.

Land cover class	Description
Cloud	Cloud and light-colored cloud shadow
Forest	Primary forest, secondary forest, native and exotic tree plantations, swamp forest
Other	Unclassified pixels
Pasture	Open to woody grassy pasture
Row crop	Large monocultures of banana, sugarcane, heart-of-palm (palmito), pineapple, coffee
Urban	Residential, commercial and industrial services, transportation, bare soil, beaches, sandy areas, bare exposed rock, quarries, mines
Water	Streams, rivers, canals, lakes, reservoirs, bays, lagoons, estuaries, dark-colored cloud shadows

Table 1.3: Summary of land cover class area statistics for six watersheds in 2001.

Land cover class	Chirripo		Tortuguero		Reventazón		Pacuare		Bananito		Estrella	
	(km^2)	(%)	(km^2)	(%)	(km^2)	(%)	(km^2)	(%)	(km^2)	(%)	(km^2)	(%)
Unclassified	6	0	1	0	2	0	0	0	0	0	1	0
Water	95	2	16	1	16	1	7	1	2	1	7	1
Forest	3177	74	712	57	1477	59	572	79	155	80	850	86
Row Crop	93	2	103	8	111	4	51	7	11	6	25	3
Pasture	773	18	351	28	642	26	73	10	16	8	76	8
Urban	158	4	70	6	271	11	25	3	7	3	13	1
Cloud	4	0	2	0	0	0	0	0	2	1	11	1
Other	1	0	0	0	0	0	0	0	0	0	0	0

Table 1.4: Summary of land cover class area statistics for six watersheds in 2014.

Land cover class	Chirripo		Tortuguero		Reventazón		Pacuare		Bananito		Estrella	
	(km^2)	(%)	(km^2)	(%)	(km^2)	(%)	(km^2)	(%)	(km^2)	(%)	(km^2)	(%)
Unclassified	6	0	1	0	1	0	0	0	0	0	1	0
Water	66	2	8	1	14	1	4	1	1	1	4	0
Forest	3155	73	673	54	1459	58	552	76	164	85	895	91
Row Crop	191	4	131	10	109	4	42	6	15	8	28	3
Pasture	672	16	355	28	571	23	85	12	9	5	36	4
Urban	217	5	87	7	366	15	44	6	4	2	19	2

Table 1.5: Difference in area from 2001 to 2014 for each land cover class for six watersheds.

Land cover class	Chirripo		Tortuguero		Reventazón		Pacuare		Bananito		Estrella		Total	
	(km^2)	(%)	(km^2)	(%)	(km^2)	(%)	(km^2)	(%)	(km^2)	(%)	(km^2)	(%)	(km^2)	(%)
Water	-30	-31	-7	-49	-2	12	-3	37	-1	-65	-3	-42	-46	-48
Forest	-22	-1	-39	-5	-19	-1	-21	-4	10	6	46	5	-45	-1
Row Crop	98	106	28	28	-2	-2	-8	-16	4	32	4	14	123	24
Pasture	-101	-13	4	1	-71	-11	12	17	-7	-46	-40	-52	-203	-12
Urban	59	37	17	24	95	35	19	78	-3	-41	5	38	193	26

Table 1.6: Matrices of land cover and changes (km^2) from 2001 to 2014.

a. Chirripo

	2001								
2014	Unclassified	Water	Forest	Row Crop	Pasture	Urban	Cloud	Other	2014 Total
Unclassified	5	0	1	0	0	0	0	0	6
Water	0	48	8	1	3	5	0	0	66
Forest	1	31	2847	17	210	45	3	0	3155
Row Crop	0	1	57	53	63	18	0	0	191
Pasture	0	6	179	13	429	45	0	0	672
Urban	0	10	85	9	68	45	0	1	217
2001 Total	6	95	3177	93	773	158	4	1	4307

b. Tortuguero

	2001								
2014	Unclassified	Water	Forest	Row Crop	Pasture	Urban	Cloud	Other	2014 Total
Unclassified	0	0	0	0	0	0	0	0	1
Water	0	5	2	0	0	0	0	0	8
Forest	0	9	589	12	50	10	2	0	673
Row Crop	0	0	23	65	32	10	0	0	131
Pasture	0	1	77	12	238	27	0	0	355
Urban	0	1	20	13	31	22	0	0	87
2001 Total	1	16	712	103	351	70	2	0	1254

c. Reventazón

	2001								
2014	Unclassified	Water	Forest	Row Crop	Pasture	Urban	Cloud	Other	2014 Total
Unclassified	0	0	0	0	0	0	0	0	0
Water	0	6	3	0	2	3	0	0	14
Forest	1	4	1245	13	155	41	0	0	1459
Row Crop	0	1	17	39	43	9	0	0	109
Pasture	1	2	124	28	348	68	0	0	571
Urban	0	3	87	31	94	151	0	0	366
2001 Total	2	16	1477	111	642	271	0	0	2519

d. Pacuare

	2001								
2014	Unclassified	Water	Forest	Row Crop	Pasture	Urban	Cloud	Other	2014 Total
Unclassified	0	0	0	0	0	0	0	0	0
Water	0	2	1	1	0	0	0	0	4
Forest	0	3	525	3	16	5	0	0	552
Row Crop	0	0	6	31	4	2	0	0	42
Pasture	0	1	25	7	43	8	0	0	85
Urban	0	1	16	9	9	9	0	0	44
2001 Total	0	7	572	51	73	25	0	0	728

e. Bananito

	2001								
2014	Unclassified	Water	Forest	Row Crop	Pasture	Urban	Cloud	Other	2014 Total
Unclassified	0	0	0	0	0	0	0	0	0
Water	0	0	0	0	0	0	0	0	1

Forest	0	1	147	1	11	3	2	0	164
Row Crop	0	0	2	10	1	2	0	0	15
Pasture	0	0	4	0	3	1	0	0	9
Urban	0	0	2	0	1	1	0	0	4
2001 Total	0	2	155	11	16	7	2	0	192

f. Estrella

	2001								
2014	Unclassified	Water	Forest	Row Crop	Pasture	Urban	Cloud	Other	2014 Total
Unclassified	1	0	0	0	0	0	0	0	1
Water	0	2	2	0	0	1	0	0	4
Forest	0	4	824	3	47	6	11	0	895
Row Crop	0	0	4	18	4	2	0	0	28
Pasture	0	1	12	2	20	3	0	0	36
Urban	0	1	8	2	5	3	0	0	19
2001 Total	1	7	850	25	76	13	11	0	983

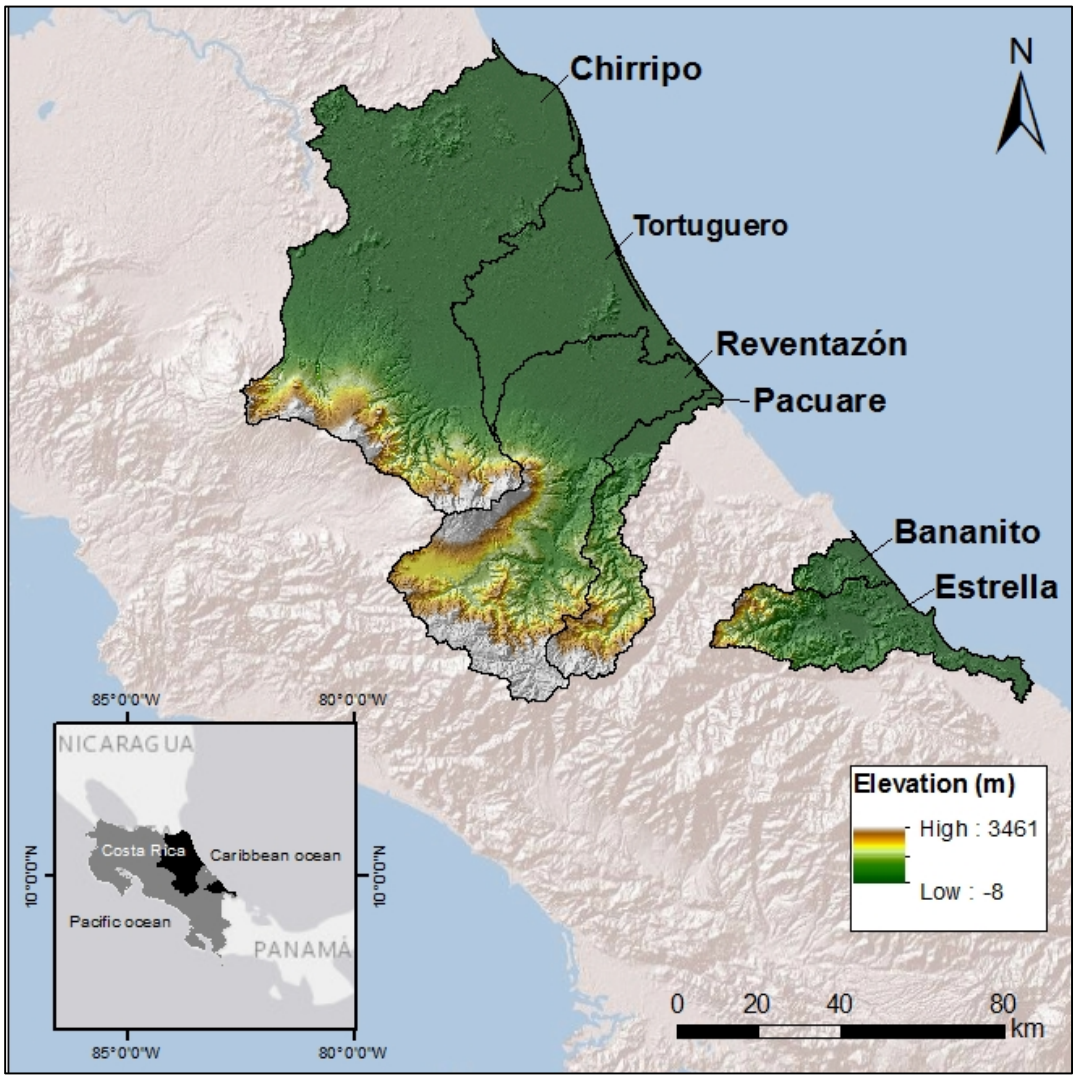

Figure 1.1: Location of the six study watersheds on the Caribbean coast of Costa Rica.

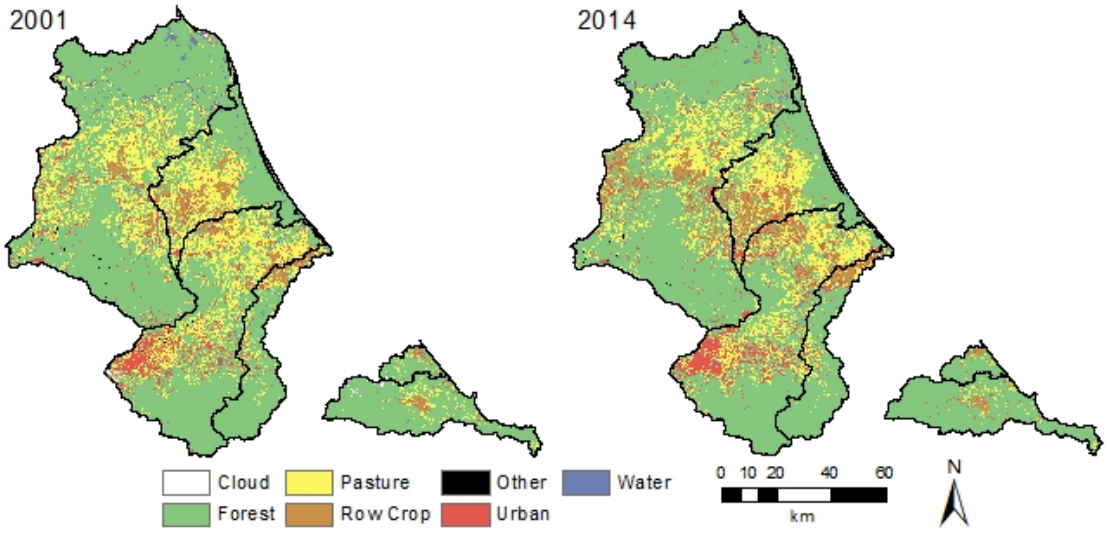

Figure 1.2: Land cover classification maps for 2001 and 2014 for the six watersheds on the Caribbean coast of Costa Rica.

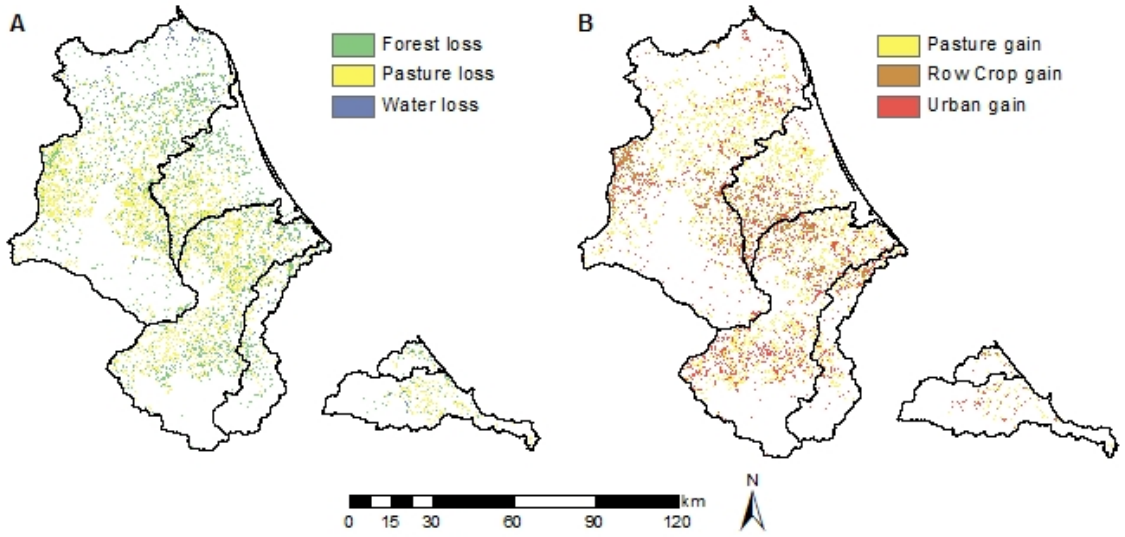

Figure 1.3: Loss in forest, pasture, and water land cover classes (A) and gain in pasture, row crop, and urban land cover classes (B) from 2001 to 2014.

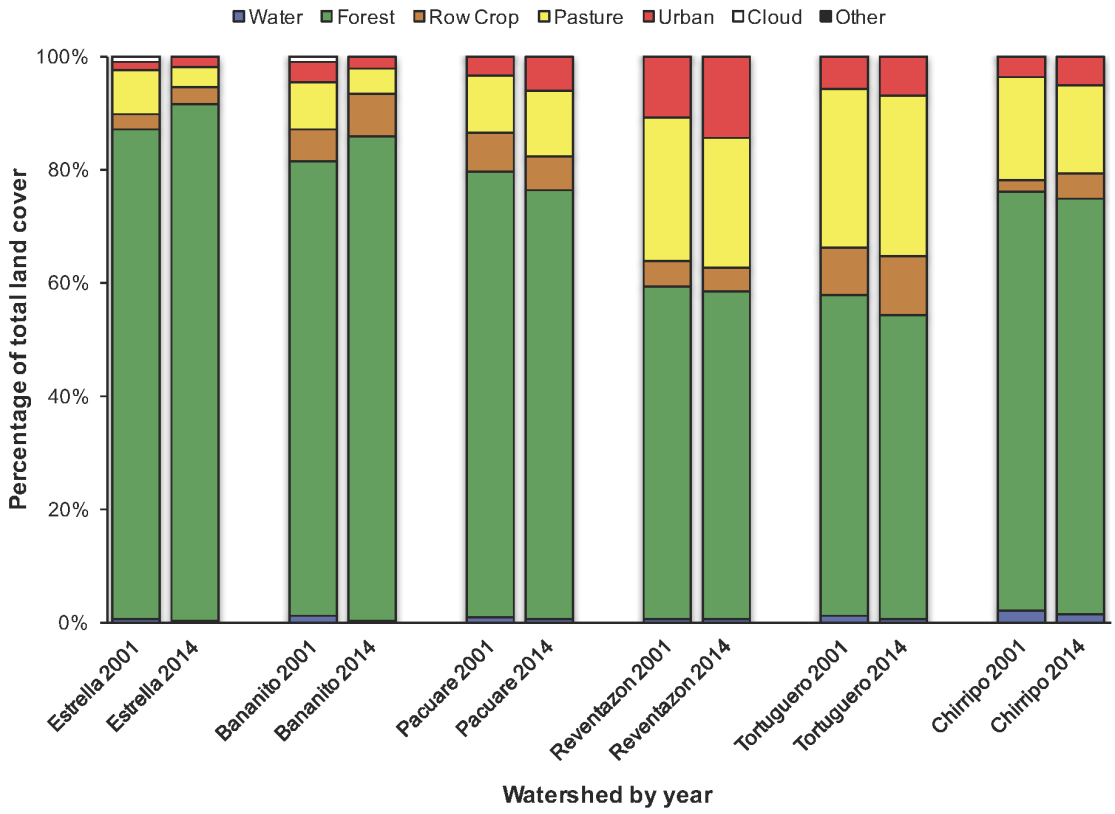

Figure 1.4: Summary of percent total land cover area by class for six watersheds in 2001 and 2014.

CHAPTER 2: Use of Chironomidae (Diptera) Surface-Floating Pupal Exuviae as a Rapid Bioassessment Protocol for Water Bodies

Summary

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.

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 (Southerland and Stribling 1995). Traditional methods of sampling benthic macroinvertebrates for RBPs, such as the dip-net, Surber sampler, and Hess sampler (Merritt et al. 2008), can be time-consuming, expensive, and may only measure assemblages from a particular microhabitat (Ferrington et al. 1991). An efficient, alternative RBP for generating biological information about a particular water body involves collection of Chironomidae surface-floating pupal exuviae (SFPE) (Ferrington et al. 1991).

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 (Ferrington 2008). Chironomids are useful in documenting water and habitat quality in many aquatic ecosystems because of their high diversity and variable pollution tolerance levels (Ferrington et al. 2008). 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 (Armitage et al. 1995; Ferrington et al. 2008). Following emergence of the terrestrial adult, the pupal exuviae (cast pupal skin) remains floating on the water's surface (Fig. 2.1). 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 (Coffman 1973).

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 (Ferrington et al. 2008). 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 (Ferrington et al. 1991); (4) collecting, processing, and identifying samples is efficient and economical in comparison to traditional sampling methods (Anderson and Ferrington 2011; Bouchard and Ferrington 2011; Ferrington et al. 1991); (5) accumulated exuviae represent taxa that have originated from a wide range of microhabitats (Wilson 1994); (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 (Wentsel et al. 1978).

The SFPE method is not a new approach for gathering information about chironomid communities. Use of SFPE was first suggested by Thienemann in the early 1900s (Thienemann 1910). A variety of studies have used SFPE for taxonomic surveys (*e.g.,* Anderson et al. (2014); Brundin (1966); Coffman and de la Rosa (1998)), biodiversity and ecological studies (*e.g.,* Andersen and Sæther (2007); Anderson and Ferrington (2012a); Bouchard and Ferrington (2009); Coffman (1973); Hardwick et al. (1995)), and biological assessments (*e.g.,* Raunio et al. (2007); Ruse (2011); Wilson and Bright (1973)). 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*., Anderson and Ferrington (2011); Bouchard and Ferrington (2011); Rufer and Ferrington (2008)). 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 (2011) determined that based on a 100-count subsample, $1/3^{rd}$ 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. 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 (Ferrington et al. 1991).

Importantly, the SFPE method has been standardized in the European Union

(known as chironomid pupal exuviae technique (CPET)) (CEN 2006) and North America (Ferrington 1987) for ecological assessment, but the method has not been described in detail. One application of the SFPE methodology was described by Ferrington et al. (1991); 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.

Materials and Methods

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 Appendix C).

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) (Fig. 2.2A), 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 (Fig. 2.2B). 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.

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 (2011) 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.

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.

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 (Fig. 2.3).
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 (Fig. 2.4A).
    1. Split the cephalothorax along the ecdysial suture (Fig. 2.4B) and open the cephalothorax so that the suture edges are on opposite sides (Fig. 2.4C).
    2. Orient the cephalothorax so that the ventral side is facing up (Fig. 2.4C).
    3. Position the abdomen dorsal side up; place immediately below the cephalothorax (Fig. 2.4C).
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.

Genus Identification

1. Determine genus of slide-mounted specimens using a compound microscope. Identify specimens to genus using keys and diagnoses in Wiederholm (1986) and Ferrington et al. (2008). If needed, confirm family-level identification using Ferrington et al. (2008). NOTE: There have been numerous generic descriptions and revisions since Wiederholm (1986) and Ferrington et al. (2008); therefore, these keys and diagnoses are incomplete and need to be supplemented with primary literature.

Results

Figure 2.1 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 2.2A. 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 2.2B. 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 (Ferrington 1987; Ferrington et al. 1991). For example, Ferrington et al. (1991) 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 (Ferrington et al. 1991).

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 2.3. 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 2.4A 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 2.4B 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 2.4C 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 (Fig. 2.5A) and genus richness (Fig. 2.5B) and cumulative species composition along a gradient of mean phosphorus concentration/mean lake depth (Fig. 2.6) (Rufer and Ferrington 2008). 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 (2008) determined that four SFPE samples per lake per season recovered the majority of the chironomid community and detected important seasonal variation in urban lakes (Fig. 2.5A, 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.

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 (Fig. 2.2A); (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 (Fig. 2.4A); and (4) recognizing key morphological characters of chironomid pupal exuviae to correctly identify to genus.

Detecting areas of high SFPE accumulation (Fig. 2.2A**)** 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" (Wilson and Ruse 2005). 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 (Fig. 2.4A**)**. 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 (1986) and Ferrington et al. (2008) 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 (Egan 2014). For sampling frequency recommendations, see Bouchard and Ferrington (2011) for streams and Rufer and Ferrington (2008) 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 (Wilson and Ruse 2005). Field evidence suggests that fifty percent or more of the exuviae do not displace more than 100 meters downstream of where the adult emerges (Wilson and Bright 1973). 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 (Ferrington 1987). 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 (Raunio et al. 2011);

(2) the inability to assess major lifecycle events and instar duration prior to eclosion, since voltinism is often challenging to determine (Coffman 1973); (3) strong seasonal variability to assemblages detected (Wilson and Ruse 2005); (4) a bias against species with lightly chitinized exuviae that break down or sink at a faster rate (Kavanaugh et al. 2014); (5) not being able to identify specimens to species if pupae and adult males have not previously been associated (Ferrington et al. 2008); 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 (Ferrington et al. 2008). 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 (Anderson et al. 2013a; Ekrem and Willassen 2004; Ekrem et al. 2007).

Conclusion

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.

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Tables and Figures

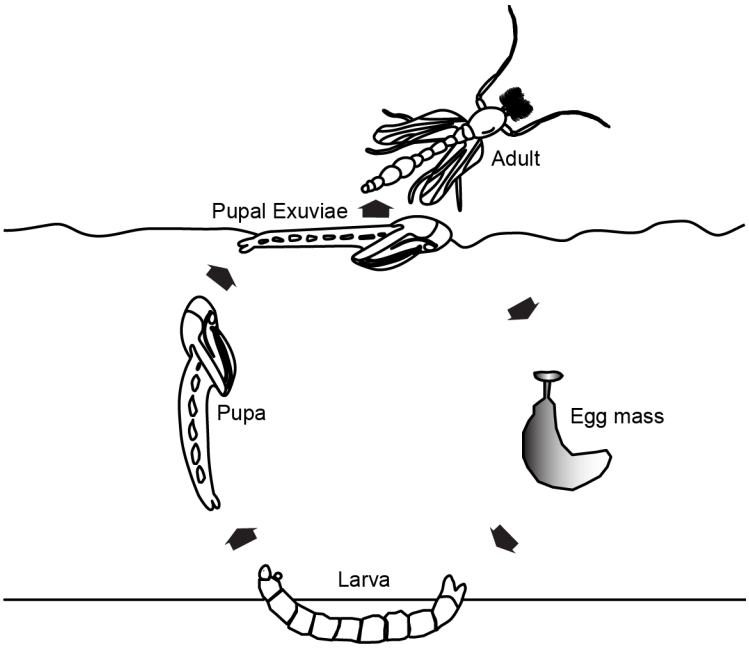

Figure 2.1: Chironomid life cycle. There are four life stages, egg, larva, pupa, and adult, in the chironomid life cycle. Female adults lay eggs on the surface of the water. Eggs sink to the bottom and typically hatch in several days to one week. After leaving the egg mass, larvae burrow into the mud 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.

Figure 2.2: 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.

Figure 2.3: Diagram showing locations of slide date and locality label (left), identification label (right), and slide mounted pupal exuviae under coverslip (center).

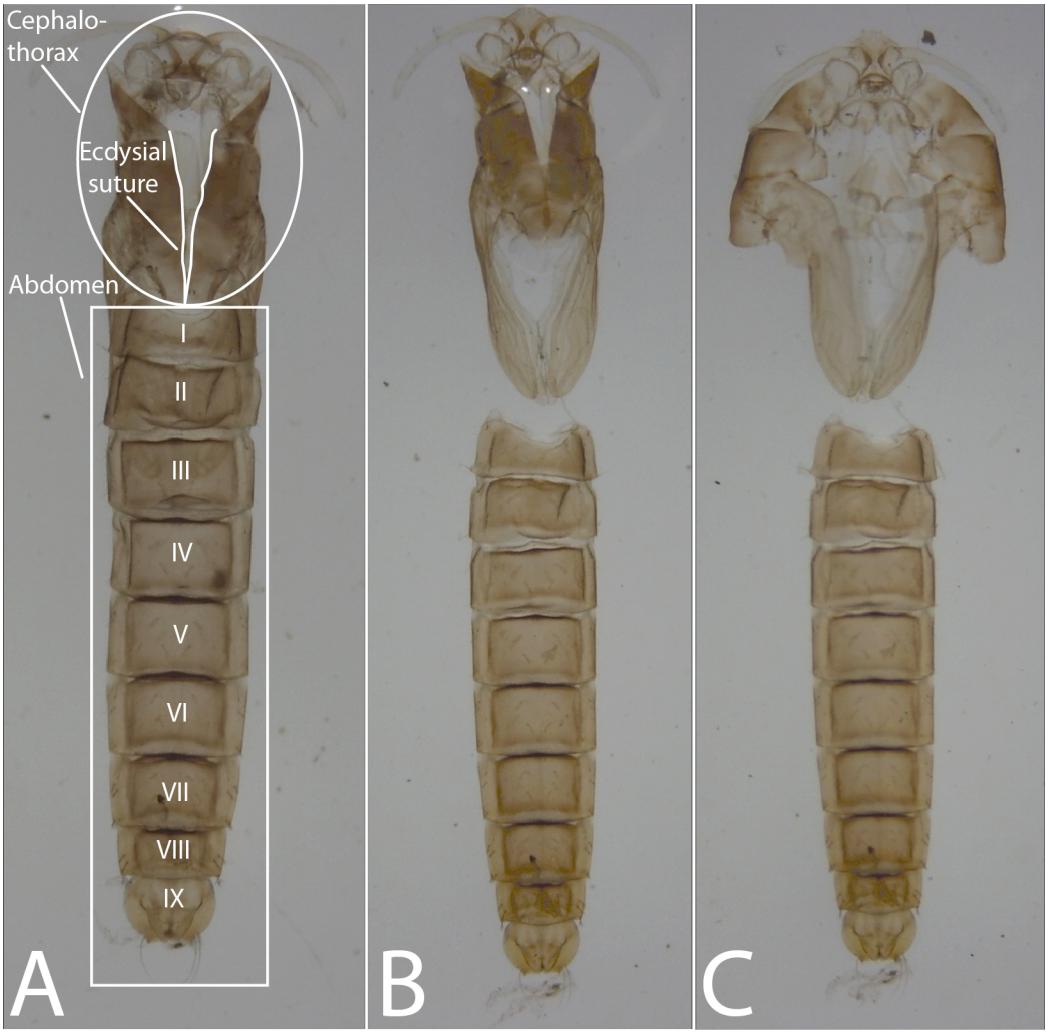

Figure 2.4: Step-by-step pupal exuviae dissection and orientation. (A) Undissected pupal exuviae (cephalothorax and abdomen with segments numbered in dorsal view). (B) Dissected pupal exuviae (cephalothorax and abdomen in dorsal view). (C) Dissected and oriented pupal exuviae (cephalothorax: ventral view; abdomen: dorsal view).

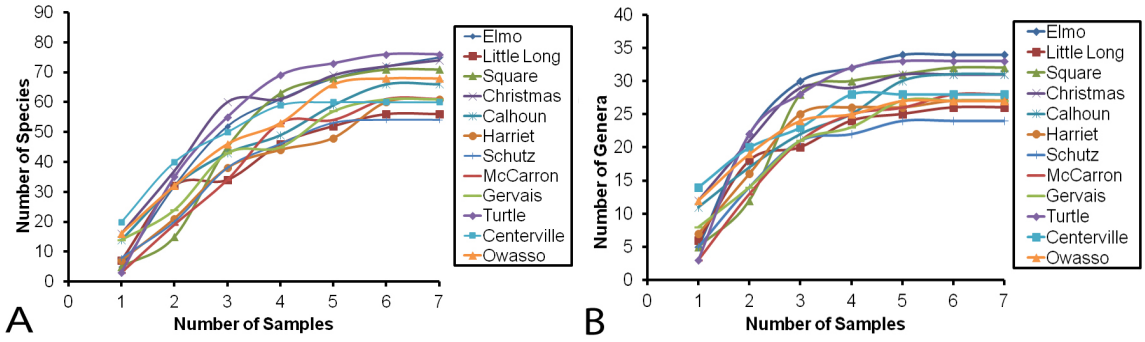

Figure 2.5: Taxonomic accumulation curves for SFPE samples collected from 16 urban lakes in Minnesota. For both panels, each colored line represents one of the 16 lakes. See Rufer and Ferrington (2008) for a detailed description of the characteristics of each lake. Each data point represents a monthly 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.

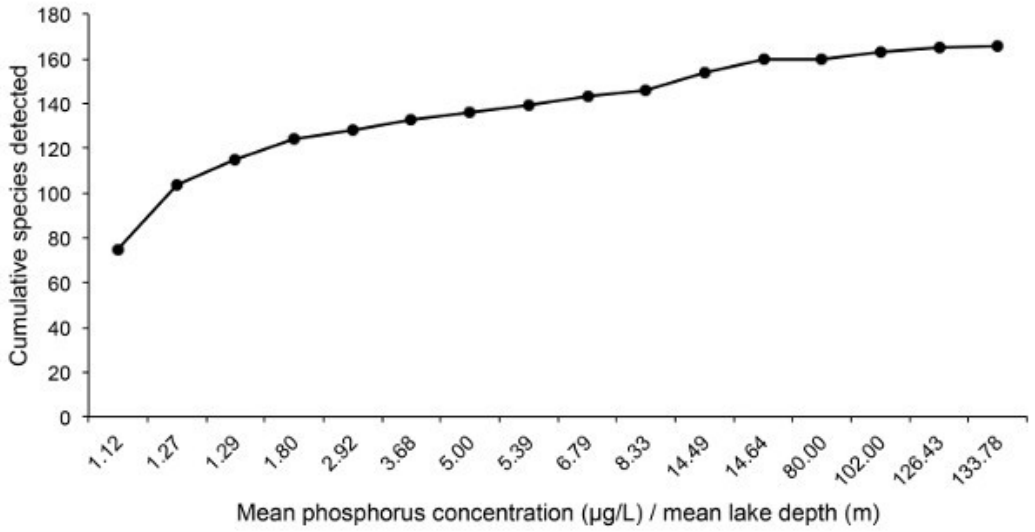

Figure 2.6: Cumulative species detected across a gradient of lake chemistries from multiple SFPE samples as a function of mean epilimnetic phosphorus concentration

(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 (2008) 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.

CHAPTER 3: Chironomidae (Diptera) species diversity of estuaries across a land use gradient on the Caribbean coast of Costa Rica

Summary

Land conversion of native rainforest ecosystems to monoculture plantation agriculture has posed significant risks to water quality of Neotropical estuaries in Costa Rica. Chironomidae assemblages are likely to provide a useful measure of biotic integrity in Neotropical estuaries of Costa Rica, which lack an intensive estuarine bioassessment tool to support environmental monitoring. The objectives of this research were to (1) characterize the taxonomic composition of Chironomidae in Neotropical estuaries on the Caribbean coast of Costa Rica, (2) test a Chironomidae Index of Biotic Integrity (CIBI) for evaluating the surface water quality and physical habitat of Neotropical estuaries, and (3) make recommendations for increasing the sensitivity of the CIBI to detect differing degrees of stress across a range of Neotropical estuaries. I identified 228 morphospecies and 70 genera from 17,071 Chironomidae surface-floating pupal exuviae collected from nine estuaries on the Caribbean coast of Costa Rica. The estuaries ranked in the following order from lowest to highest biotic integrity based on CIBI scores: Estero Negro (14), Laguna Cuatro (43), Laguna Jalova (49), Laguna del Tortuguero (50), Río Parismina (51), Laguna Barra del Colorado (57), Río Pacuare (59), Río Bananito (64), and Río Estrella (71). The CIBI successfully differentiated between estuaries with poor to good biotic integrity, which indicates that CIBI can be used to evaluate the surface water quality and physical habitat of Neotropical estuaries. I recommend that future studies

adopt my approach and refine it by developing regionally accurate genus and corresponding species-level tolerance values to improve the sensitivity of the CIBI for biological monitoring of Neotropical estuaries.

Introduction

During the past several decades, agricultural intensification has increased nutrient enrichment and caused widespread eutrophication, accelerating the flow of nutrients to estuaries and other coastal marine ecosystems (ECEs) (Bricker et al. 2008; Howarth et al. 2011; Kennish and Townsend 2007). Nutrient over-enrichment has been identified as the prime cause of water quality and habitat degradation of ECEs (Boesch et al. 2001; Nixon 1995; Paerl et al. 2014). In tropical regions, land conversion of native rainforest ecosystems to monoculture plantation agriculture has posed significant risks to ECEs (Downing et al. 1999; Kress et al. 2002; Lovelock et al. 2004).

Banana and pineapple plantations are two types of agricultural land uses that impact ECEs on the Caribbean coast of Costa Rica (Castillo et al. 2006; Echeverría-Sáenz et al. 2012; Grant et al. 2013). Both types of monoculture agriculture are linked to water pollution via modifications in water movement through the construction of canals that channelize runoff, and application of large quantities of agrochemicals through aerial spraying and ground application (Castillo et al. 1997; Castillo et al. 2000; Diepens et al. 2014; Pringle et al. 2016). In addition, the Caribbean coast of Costa Rica is experiencing urbanization by increasing the number of paved and improved gravel roads to accommodate the expansion of pineapple and banana plantations (Fagan 2014).

Kranzfelder et al. (in review-a) found that expansion of agricultural plantations was the most pronounced land use change in the northeastern Caribbean watersheds of Chirripo and Tortuguero, while urbanization was the most evident change in the south-central watersheds of Reventazón and Pacuare.

Biological multimetric indices, such as the Index of Biotic Integrity (IBI), are effective and efficient bioassessment tools that utilize several metrics, including taxa richness, taxonomic composition, and taxa tolerance/intolerance, to evaluate the impact of multiple stressors on the health of ECEs (Barbour et al. 1999; Herman and Nejadhashemi 2015; Karr 1981; Weisberg et al. 1997). Traditional multimetric IBI approaches have focused on periphyton, benthic macroinvertebrate, and fish assemblages (Barbour et al. 1999; Karr 1981; Karr et al. 1986). However, the family Chironomidae (Diptera), commonly referred to as non-biting midges or chironomids, is the most widely distributed, most diverse, and often the most abundant of all families of benthic macroinvertebrates in aquatic ecosystems, including ECEs (Ferrington 2008). Chironomid communities are considered valuable bioindicators of water quality due to their high species diversity and the varying sensitivity of these species to land use changes, including urbanization, agriculture, and deforestation (Lunde and Resh 2012; Nicacio and Juen 2015; Rosenberg 1992; Ruse 2010; Thorne and Williams 1997). There are nearly 900 species described from the Neotropical Region (Spies et al. 2009; Spies and Reiss 1996), and up to 20,000 species may exist worldwide (Ferrington 2008). In addition, chironomids are among the few aquatic insect families that have adapted to live in a wide range of salinities, from freshwater to seawater, and can be a major component

of the fauna of brackish waters (Cañedo-Argüelles et al. 2012; Casas and Vilchez-Quero 1996; Dimitriadis and Cranston 2007; James et al. 2003; Williams and Hamm 2002; Williams and Williams 1998). For example, Kranzfelder and Ferrington (2016) identified 98 species from one brackish Costa Rican estuary, Laguna del Tortuguero, with species diversity structured along a salinity gradient.

One low-cost, easy-to-use, and efficient method of assessing chironomid communities involves collections of Chironomidae surface-floating pupal exuviae (SFPE) (Ferrington et al. 1991; Kranzfelder et al. 2015; Wilson and Ruse 2005), which is the exoskeleton shed by the adult as it emerges on the surface of the water. SFPE accumulate behind obstructions, like fallen trees or along banks, through the action of wind or water current and can be collected to give a comprehensive sample of chironomid communities for biological monitoring purposes (Kranzfelder et al. 2015). Collections of SFPE samples have many advantages over benthic samples of chironomid larvae including: (1) enhanced ease of species identification, (2) more accurate estimates of the number of species detected, (3) better collection from a range of microhabitats, including areas that are difficult to sample with other collection methods (e.g. wood, sand, and deep waters), and (4) improved measurement of how water quality characteristics influence biodiversity of aquatic systems, since SFPE collected represent individuals that have successfully completed their life cycle in that aquatic habitat (Bouchard and Ferrington 2011).

In Costa Rica, the Biological Monitoring Working Party-Costa Rica (BMWP-CR) biotic index was adopted in the Executive Decree No. 33902-S-MINAE (Ministerio de

Ambiente y Energía, Propuesta de Ley del Recurso Hídrico, 2007) for assessment of environmental quality of waters (Gutierrez-Fonseca and Lorion 2014; Maue and Springer 2008; Rizo-Patrón V et al. 2013; Stein et al. 2008). The BMWP-CR index value is based on the presence of macroinvertebrate families and their tolerance scores. According to their protocol, Chironomidae have a score of 2.0 and are categorized as being indicators of poor water quality. However, family identification does not provide sufficient resolution for sensitive and accurate bioassessments (Bailey et al. 2001; Bouchard 2005; Lenat and Resh 2001), since tolerance to pollution varies by genus (Ruse 2002; Wilson and Ruse 2005), and in several instances among species within a genus. For example, *Cricotopus*, *Chironomus*, and *Dicrotendipes* are tolerant of organic pollution, while *Cladotanytarsus*, *Parametriocnemus*, and *Paralauterborniella* are intolerant of organic pollution (Wilson and Ruse 2005). As a result, Chironomidae IBIs have been developed for bioassessment of wadeable groundwater- and surface-water-dominated streams in Minnesota (Bouchard and Ferrington 2011) and wadeable perennial streams in the Northern Glaciated Plains ecoregion (Kafle 2013). Thus, Chironomidae assemblages are likely to provide a useful measure of biotic integrity in Neotropical estuaries of Costa Rica, which lack an intensive estuarine bioassessment tool to support environmental monitoring and regulatory programs.

The objectives of this research were to (1) characterize the taxonomic composition of Chironomidae in Neotropical estuaries on the Caribbean coast of Costa Rica, (2) test a Chironomidae Index of Biotic Integrity (CIBI) for evaluating the surface water quality and physical habitat of Neotropical estuaries, and (3) make

recommendations for increasing the sensitivity of CIBI to detect differing degrees of stress across a range of ECEs.

Materials and Methods

Sample area

I selected nine estuaries within six different watersheds across a land use gradient located on the Caribbean coast of Costa Rica (Table 3.1, Fig. 3.1). These six watersheds were selected to represent a land use gradient from mostly primary and secondary tropical rainforest to largely monoculture plantation agriculture (e.g. banana and/or pineapple). In the city of Limon (09º57 N, 83º01 W; elevation 5m) (Fig. 3.1), the mean monthly temperature is 25.9ºC and the mean total monthly rainfall is 298.3 mm. Mean total monthly precipitation is 436.1 mm over an average of 22 rainy days in July and 317.0 mm over an average of 19 rainy days in January (Instituto Meteorológico Nacional 2016). See Kranzfelder et al. (in review-a) for a detailed description of the study area, land uses, and land covers of the six watersheds.

Data collection and processing

I collected data biannually for two consecutive years (July 2012, Jan. 2013, July 2013, Jan. 2014). I sampled each of the nine estuaries one day during each sample event. I collected data from three zones in each estuary that represent a range of estuarine conditions: (1) the transition of the river into the estuary (1-10 ppt), (2) the middle of the estuary (10-20 ppt), and (3) near the outlet of the estuary to the ocean (20-30 ppt). I

collected SFPE samples along 500-1,000 meter reaches of the left and right descending banks at each sample zone. I used a YSI Pro2030 field dissolved oxygen/conductivity handheld meter to measure the following physiochemical parameters from each of the three zones: water temperature, dissolved oxygen, and salinity. I followed standard protocol using a Secchi disk to measure water clarity (Table 3.1).

Chironomidae SFPE samples were collected from both banks of each zone to account for hourly-to-daily changes in wind and tidal pattern, which may affect the down water drift of chironomid pupal exuviae. I collected 24 SFPE samples per estuary (3 zones x 2 SFPE samples/zone x 4 sample events) and followed methods described by Kranzfelder et al. (2015), except samples were collected for 20 minutes instead of 10 minutes, as suggested by Siqueira et al. (2008) and Kranzfelder and Ferrington (2016). Briefly, we collected SFPE samples by dipping a white plastic tray into areas of known SFPE accumulation (e.g. behind a fallen tree). I poured contents from the tray through a 125-µm-aperture US Standard test sieve to retain chironomid SFPE and residue. I transferred the sample to 60-mL jars and preserved with 95% ethanol.

In the laboratory, I placed a small portion of the sample in a Syracuse dish and examined it under a dissecting microscope. Next, I picked chironomid SFPE from the sample into 1-dram vials with 80% ethanol. After looking for all SFPE in the sample dish, we swirled the Syracuse dish and look for any remaining pupal exuviae stuck to the sides of the dish. I repeated this swirling step two times to recover any additional SFPE. I picked the entire sample of SFPE, but did not pick whole pupae, SFPE with adults still

attached to the pupal exuviae, or broken specimens with less than three-fourths of the specimen complete to avoid species identification problems.

I chose a subsample size of 500 specimens to be slide mounted. In temperate streams, a subsample size of 300 specimens was on average sufficient to identify a large proportion (85%) of the Chironomidae community (Bouchard and Ferrington 2011). However, the larger subsample size was chosen to ensure that SFPE samples represent a large proportion of the Chironomidae community from the relatively unknown ecosystem of Neotropical estuaries. I dehydrated specimens in 95% ethanol, dissected, and slide mounted in Euparal. Then, I made species identifications of slide-mounted specimens under a compound scope to morphospecies or the lowest taxonomic level possible. All contemporary references were checked, but the following were especially useful: Beck and Beck (1966), Boesel (1974), Paggi (1977), Roback (1980), Roback and Coffman (1983), Borkent (1984), Sawedal (1984), Wiederholm (1986), Roback (1986), Soponis (1987), Epler (1988a), Langton (1991), Caldwell (1993), Sublette and Sasa (1994), Serrano and Nolte (1996), Wiedenbrug and Fittkau (1997), Epler and Janetzky (1998), Hestenes and Saether (2000), Jacobsen and Perry (2000), Andersen and Mendes (2002), Wiedenbrug and Andersen (2002), Mendes et al. (2003), Sæther (2004), Wiedenbrug and Ospina-Torres (2005), Ekrem (2007), Ferrington et al. (2008), Jacobsen (2008), Tejerina and Paggi (2009), Wiedenburg et al. (2009), Wiedenburg and Trivinho-Strixino (2009), Oliveira et al. (2010), Trivinho-Strixino (2010), Ferrington and Saether (2011), Oliveira and Silva (2011), Saether (2011), Wiedenburg and Trivinho-Strixino (2011), Kranzfelder (2012), Sæther and Cranston (2012), Wiedenbrug et al. (2012), De Oliveira et al. (2013),

Wiedenburg et al. (2013), Da Silva et al. (2014), Donato et al. (2015), Silva and Ekrem (2016), and Tang (2016). Finally, I deposited voucher specimens in the insect collections at the University of Minnesota (UMSP) and University of Costa Rica (UCR).

Data analysis

I pooled 24 chironomid SFPE samples for each of the nine estuaries. First, I estimated theoretical species richness in EstimateS with the abundance-based Chao 1 classic richness estimator (Eq. 1), which is a nonparametric species-richness estimator that takes into account relative abundance and performs well on data that include many rare species (Chao 1984; Colwell 2013):

$$S_{est} = S_{obs} + a^2/2b \quad (1)$$

Where S_{obs} = the number of observed species in the sample; a = the number of species represented by only 1 individual; and b = the number of species represented by 2 individuals.

Second, based on recommendations by Bouchard and Ferrington (2011) for Minnesota streams, I tested an "extrinsic" Chironomidae Index of Biotic Integrity (CIBI) using 10 community composition metrics and diversity and biotic indices: Total Species Richness, Berger-Parker Index of Dominance (Eq. 2), Shannon's Diversity Index (Eq. 3), and Margalef's Diversity Index (Eq. 4), Hilsenhoff's Biotic Index (Eq. 5), % Tolerant Genera, % Intolerant Genera, % Orthocladiinae, % Chironomini, and % Tanytarsini. Although these benthic metrics were regionally developed, they are effective at measuring a response across a range of human influence over a wide geographic area

(Barbour et al. 1999). Berger-Parker Index of Dominance was calculated as:

$$D_{BP} = \frac{n_{max}}{N} \quad (2)$$

Where n_{max} = the number of specimens for the most common species and N = the total number of specimens in the sample (Berger and Parker 1970; Magurran and McGill 2011). Shannon's Diversity Index was calculated as:

$$H' = -\sum \left(\frac{n_i}{N}\right) \ln \left(\frac{n_i}{N}\right) \quad (3)$$

Where n_i = the number of specimens for the *i*th species and N = the total number of specimens in the sample (Magurran and McGill 2011). Margalef's Diversity Index was calculated as:

$$D_{MG} = \frac{(S-1)}{\ln N} \quad (4)$$

where S = total number of species in the pooled sample and N = total number of specimens in the sample. Hilsenhoff's Biotic Index was calculated as:

$$HBI = \frac{\sum n_i T_i}{N} \quad (5)$$

where n_i = the number of specimens for the *i*th genus; T_i = the tolerance value for the *i*th genus; and N = the total number of specimens in the sample (Hilsenhoff 1977; Hilsenhoff 1987). I derived extrinsic tolerance values for Chironomidae used in the HBI from Barbour et al. (1999) and Ferrington et al. (2008) (Table 3.2), since tolerance values are not available for Costa Rica. When available, I used Southeast United States genus values, but for cases where these values were not provided, I used tribe or subfamily values. One exception to this protocol was *Polypedilum*, which in the Southeast United States has a genus tolerance value of 6.7 but species tolerances vary from 4.0 to 9.2.

Based on the average high metric scores for Río Bananito and Río Estrella and average low metric scores for Estero Negro, I opted to use the lowest *Polypedilum* species tolerance value of 4.0 for the numerically dominant species in Río Bananito and Río Estrella and the highest *Polypedilum* species tolerance value of 9.2 for the numerically dominant (but different) species in Estero Negro. Percent tolerant genera were calculated as the total genera out of all genera in the pooled samples with a tolerance value above 7. Percent intolerant genera were calculated as the total genera out of all genera with tolerance values below 3.

After calculating the 10 community composition metric and diversity and biotic index values for each estuary (Tables 3.5-3.6), I scored each metric from 1 to 10 points based on its response to disturbance (Tables 3.7-3.8). Metrics that respond negatively to disturbance will have metric scores positively correlated to metric values. Metrics that respond positively to disturbance will have metric scores inversely related to metric values. Metric scoring was based on ranged observed in my data set, and therefore, "intrinsic" for this study. Then, I calculated the CIBI score for each estuary sample by summing these ten metric scores. Using this approach, theoretical CIBI scores for each estuary range from 10 to 100. Lower IBI scores indicate lower biotic integrity ratings and imply higher human disturbance (Table 3.3) (Karr et al. 1986). See Appendix F for a detailed description of each metric.

Results

Percent forest land cover per watershed ranged from 53.7% for Tortuguero watershed to 91.0% for Estrella watershed. Average water temperature ranged from 24.7ºC for Río Estrella to 27.5 ºC for Estero Negro. Average dissolved oxygen ranged from 1.7 mg/L for Laguna Cuatro to 7.2 mg/L for Río Estrella. Average salinity ranged from 0.1 ppt for Laguna Barra del Colorado and Río Parismina to 13.0 ppt for Laguna del Tortuguero (Table 3.1).

I identified 228 morphospecies and 70 genera from 17,071 pupal exuviae contained in the samples. Most of the morphospecies (220) and 14 genera are either undescribed or unknown for the pupal life stage. *Tanytarsus* was the most species-rich genus with 24 morphospecies followed by *Polypedilum* with 19 morphospecies and *Cricotopus* with 13 morphospecies (Appendix D). The taxa collected included members from five subfamilies/tribes: Pseudochironomini (1.3%), Tanypodinae (3.5%), Orthocladiinae (26.8%), Tanytarsini (28.4%), and Chironomini (40.1%). Chironomini had the highest genus richness ranging from 5 to 21 genera per estuary and was followed by Orthocladiinae with 4 to 11 genera per estuary. Pseudochironomi was represented by the lowest genus richness with one genus: *Pseudochironomus* (Table 3.4). The five most abundant genera, *Tanytarsus* (20.0%), *Nanocladius* (14.1%), *Polypedilum* (12.9%), *Cricotopus* (9.5%), and *Cladotanytarsus* (5.8%), collectively accounted for 62% of all specimens present in the samples (Appendix E).

Río Estrella had the highest species richness, genus richness, and relative abundance (120, 43, and 3,620, respectively). Estero Negro had the lowest species richness, genus richness, and relative abundance (26, 13, 423, respectively) (Fig. 3.2).

Species-accumulation curves reached saturation for Laguna Cuatro, Laguna del Tortuguero, Río Bananito, and Estero Negro with an average of 80.1% of estimated species collected. However, Laguna Barra del Colorado, Laguna Jalova, Río Parismina, Río Pacuare, and Río Estrella did not reach saturation with an average of 59.7% of estimated species collected (Fig. 3.3).

Chironomidae tolerance values ranged from 1.7 to 10 ($\bar{x}$ = 6.2) (Table 3.2). Percent Orthocladiinae ranged from 2.1% to 58.0%, % Chironomini from 13.4% to 72.6%, % Tanytarsini from 4.3% to 70.1%, % Tolerant Genera from 9.5% to 28.0%, and % Intolerant Genera from 0.0% to 9.5% (Table 3.5). Total Species Richness ranged from 26 to 120, Berger-Parker Index of Dominance from 0.136 to 0.636, Shannon's Diversity Index ranged 1.71 to 3.38, Margalef's Diversity Index from 4.13 to 14.52, and Hilsenhoff's Biotic Index from 5.17 to 6.63 (Table 3.6). Río Bananito and Río Estrella scored the highest and Estero Negro the lowest on the following four Chironomidae assemblage metrics: Total Species Richness, Berger-Parker Index of Dominance, Margalef's Diversity Index, and Shannon's Diversity Index (Tables 3.7-3.8). The estuaries ranked in the following order from lowest to highest biotic integrity based on CIBI scores: Estero Negro (14), Laguna Cuatro (43), Laguna Jalova (49), Laguna del Tortuguero (50), Río Parismina (51), Laguna Barra del Colorado (57), Río Pacuare (59), Río Bananito (64), and Río Estrella (73) (Table 3.8). The five most abundant genera for the lowest CIBI score (Estero Negro) were as follows: *Polypedilum*, *Tanytarsus*, *Labrundinia*, *Dicrotendipes*, and *Cricotopus*. The five most abundant genera for the

highest CIBI score (Río Estrella) were as follows: *Tanytarsus*, *Cricotopus*, *Polypedilum*, *Cladotanytarsus*, and *Cryptochironomus* (Appendix E).

Discussion

Typically, chironomids are neglected in biological monitoring of estuarine and coastal ecosystems (ECEs) since species have been reported to typically prefer low salinities and are generally considered freshwater organisms (Pinder 1986; Williams 1998). Yet, the 228 morphospecies from 70 genera and five subfamilies/tribes demonstrates that rich chironomid communities are not only present in these Neotropical estuaries, but that species richness was high, similar to results in Kranzfelder and Ferrington (2016). Putting these results into context, I detected the equivalent of 154% of the reported 148 species of chironomids reported by Watson and Heyn (1992) during their extensive collections from lotic Costa Rican habitats, ranging from small springs to large rivers, and the equivalent of 85% of the reported 266 species of chironomids collected from 13 streams in northwestern Costa Rica (Coffman et al. 1992). Dimitriadis and Cranston (2007) recorded 44 species from 5,735 chironomid larvae collected from an Australian estuary, fewer than the number of species recorded from seven out of the nine estuaries in my study.

Species-accumulation curves reached saturation for four estuaries, including Laguna Cuatro, Laguna del Tortuguero, Río Bananito, and Estero Negro. Therefore, these results suggest that these estuaries, especially Estero Negro, were sufficiently sampled and my results are based on biotic responses, not differences in sampling effort.

However, the species-accumulation curves did not reach saturation for five estuaries, including Laguna Barra del Colorado, Laguna Jalova, Río Parismina, Río Pacuare, and Río Estrella. These five estuaries have moderate to high species richness and abundance. However, it is common for species accumulation curves to not reach an asymptote in the tropics since species diversity is high and most species are rare. For example, after nearly 30 consecutive years of sampling, an ongoing inventory of a tropical rainforest ant assemblage at La Selva, Costa Rica, has still not reached an asymptote in species richness. Tropical biodiversity studies often fall short of revealing the complete species richness for an assemblage (Gotelli and Colwell 2011). While undetected species remain in these estuaries, I argue that the rare species are undersampled and the results are based on the common species detected. I also anticipate that more extensive sampling in these Neotropical estuaries would further widen the species richness gap between the least and most species rich estuaries.

While I collected a high number of species in this study, 125 species were represented by less than 10 specimens, indicating a large number of rare species with low abundances. This pattern is consistent with other Neotropical studies involving chironomids (Coffman and de la Rosa 1998; Ferrington et al. 1993; Pringle and Hamazaki 1998; Souza et al. 2007). Coffman and de la Rosa (1998) reported low chironomid densities of less than 5,000 larvae per square meter in northwestern Costa Rican streams, compared to larval densities ranging up to 50,000 larvae per square meter reported in temperate areas (Lindegaard 1989). Presence of filter-feeding shrimp or predatory benthic macroinvertebrates and fish could account for the low abundance of

chironomids in my study estuaries. Ferrington et al. (1993) collected 2,451 chironomids from a Puerto Rican stream over the course of one year, and suggested that filter-feeding shrimp could compete with and decrease the number of filter-feeding chironomid genera, like *Rheotanytarsus*. Pringle and Hamazaki (1998) found that omnivorous fish and shrimp reduced chironomid larval densities in lowland streams of northeastern Costa Rica.

My results indicate that the CIBI I have developed can be used to evaluate the surface water quality and physical habitat of Neotropical estuaries. Individual metrics are derived from an extrinsic model and thus provide a high degree of objectivity to the CIBI. Structed in this manner, the CIBI successfully differentiated between estuaries with very poor biotic integrity (Estero Negro), estuaries with fair biotic integrity (Laguna Cuatro, Laguna Jalova, Laguna del Tortuguero, Río Parismina, Laguna Barra del Colorado, Río Pacuare), and estuaries with high biotic integrity (Río Bananito and Río Estrella).

These results, based on biotic data, follow the same patterns observed in my study based on coastal watershed land cover data, except Estero Negro (Kranzfelder et al. in review-a). In that study, I ranked watersheds in the following order from most to least impacted by human land use change, and thus, susceptible to water quality issues: Tortuguero, Reventazón, Chirripo, Pacuare, Bananito, and Estrella (Kranzfelder et al. in review-a). Estero Negro is in the Bananito watershed; and therefore, based on the land use composition, I would predict high biotic integrity, similar to Río Bananito and Río Estrella. However, on average, Estero Negro had low dissolved oxygen (3.0 mg/L) and moderate salinity (9.8 ppt) compared to the estuaries with higher biotic integrity (Table

3.1). The low dissolved oxygen could be due to high organic matter in the estuary from decomposition of plants or animals and the moderate salinity could indicate a tidally influenced estuary. I believe that for this estuary small-scale environmental factors (i.e., low dissolved oxygen and moderate salinity) had a greater impact on the taxonomic composition of the chironomid community than cumulative coastal watershed land use.

Scoring of individual metrics was based on patterns intrinsic to the study. This is preferred because ranges of metric values in tropical systems can be expected to be different than in temperate regions. Four metrics showed a large range in scores between the estuary with the highest water quality (Río Estrella) and estuary with the lowest water quality (Estero Negro): Total Species Richness, Berger-Parker Index of Dominance, Margalef's Diversity Index, and Shannon's Diversity Index. These four metrics were calculated using the species richness and relative abundance of chironomids in the samples. Therefore, Río Estrella had the highest species richness and relative abundance and scored the highest CIBI score.

By contrast, the other six metrics, which are based on the relative abundances of different tribes, relative richness of tolerant/intolerant genera using literature-based tolerance values, and abundance-weighted average of each genus using tolerance values, did not show as large a range in scores or inconsistent scores between the estuaries with lowest and highest water quality. This was especially evident when I used extrinsic genus-level values for *Polypedilum* for my initial trials related to the HBI metric. For instance, genus-level tolerance resulted in the lowest HBI score for Rio Estrella, which otherwise had moderate-to-maximum score for the other nine metrics. On closer

inspection, I realized that the numerically dominant species of *Polypedilum* in the estuary differed from numerically dominant species of *Polypedilum* in the low-ranking estuaries. Consequently, I used least and most species tolerant values to provide better sensitivity of the HBI metric and believe that these intrinsic tolerance values used are justifiable. In addition to the HBI metric, the remaining tribe- and genus-based metrics did not closely follow the trends of the four species-based metrics. Even though the 70 genera collected have a wide range of generic pollution tolerances from very intolerant (1.7) to very tolerant (10), my results suggest that there are likely to be some additional genera for which species-level differences to pollution should not be generalized by using generic tolerance values. For example, *Cladotanytarsus* has a tolerance value of 3.7 based on literature, and was very abundant in samples from both Río Estrella and Laguna Jalova. Recent research in Minnesota on lakes with differing trophic states has revealed that different species of *Cladotanytarsus* occur in oligotrophic versus hypereutrophic lakes (Ferrington, unpublished data) and should be given different tolerance values. Subsequently, differing intrinsic species tolerances for this genus may need to be developed for use in the estuarine CIBI. For example, Laguna Jalova had a higher number of individuals from this genus than Río Estrella; and so, Laguna Jalova had an HBI score of 10, while Río Estrella had an HBI score of only 3. However, the *Cladotanytarsus* present in Laguna Jalova was a different species than Río Estrella. Based on the land use in the Tortuguero watershed with 10.4% forest land cover and 28.3% pasture cover, I predict that the *Cladotanytarsus* sp. present in Laguna Jalova is

likely to be highly tolerant of organic enrichment and should have been assigned a higher intrinsic tolerance values than 3.7.

While most species were rare, there were three species-rich and abundant genera, *Cricotopus, Polypedilum*, and *Tanytarsus,* which accounted for 42.4% of the total abundance in the samples. *Cricotopus* and *Polypedilum* were collected from all nine estuaries and *Tanytarsus* was collected from all estuaries except Río Parismina. I recommend that future Neotropical estuarine biomonitoring studies focus on assessment of individual morphospecies within these three genera and develop more realistic intrinsic tolerance values of these morphospecies for Neotropical Regions, like Costa Rica. For example, the species-rich genus *Cricotopus* has some species within subgenus *Cricotopus* and subgenus *Isocladius* that are resistant to many forms of pollution, while there are some species within subgenus *Nostococladius* that are not resistant to many forms of pollution (Wilson and Ruse 2005). For genus *Polypedilum* in the Southeast USA, *Polypedilum aviceps* has a tolerance value of 4.0, while *Polypedilum illinoense* has a tolerance value of 9.2 (Barbour et al. 1999) and it can be expected that ranges in tolerance among species in Costa Rica will have similar magnitude.

Thorne and Williams (1997) have argued that genus identifications of benthic macroinvertebrates for bioassessments in tropical countries are rarely possible and propose that use of family-level biotic indices, like the qualitative BMWP-CR (Biological Monitoring Working Party-Costa Rica), is more practical. However, based on my results, I suggest that a multimetric index of biotic integrity focused on Chironomidae at genus-level is useful for evaluating the integrity of ecologically important Neotropical estuaries.

I recommend that future studies develop and evaluate regionally accurate genus, and corresponding species-level intrinsic tolerance values for *Cricotopus*, *Polypedilum*, *Cladotanytarsus*, and *Tanytarsus*, in order to improve the sensitivity of the CIBI for use in biological monitoring of ECEs in Costa Rica. In addition, morphospecies can be given a described species name by either rearing immature life stages (eggs, larvae, or pupae) to adults with species descriptions (Spies et al. 2009) or using DNA barcodes to link pupal exuviae to described species using approaches refined by Kranzfelder et al. (in review-b).

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Tables and Figures

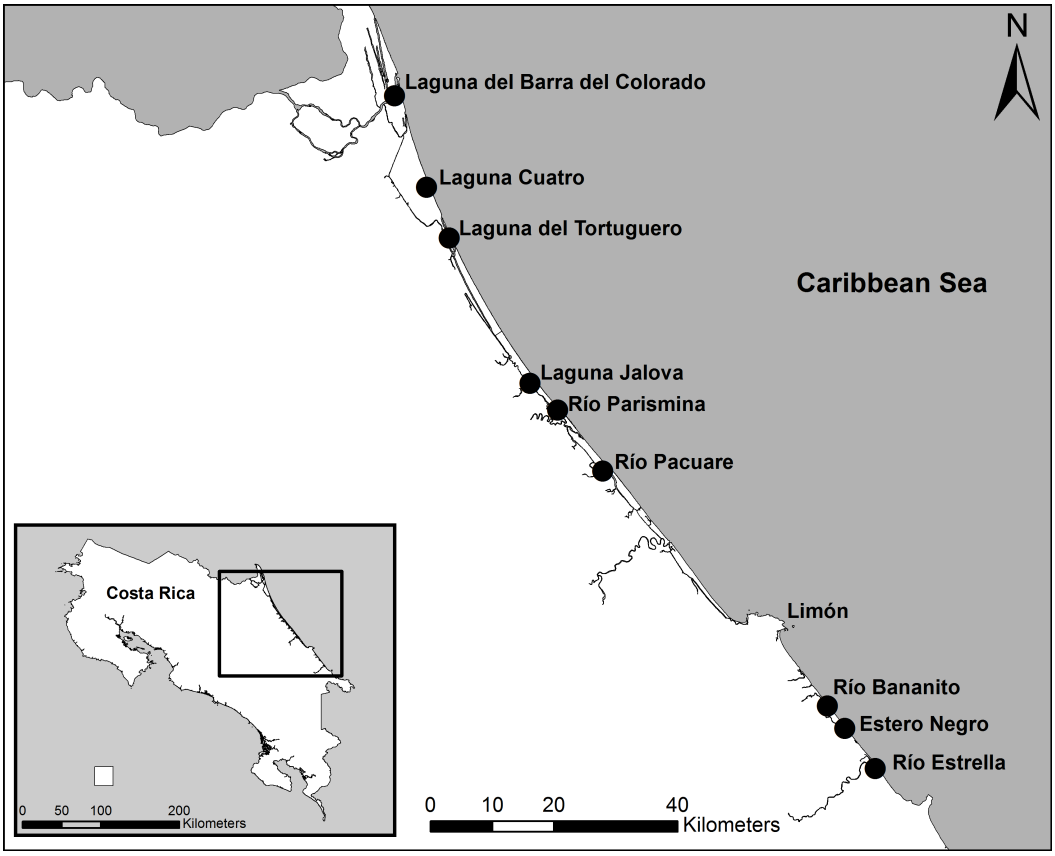

Figure 3.1: Locations of nine estuaries on the Caribbean coast of Costa Rica.

Black dot indicates location of sample estuary.

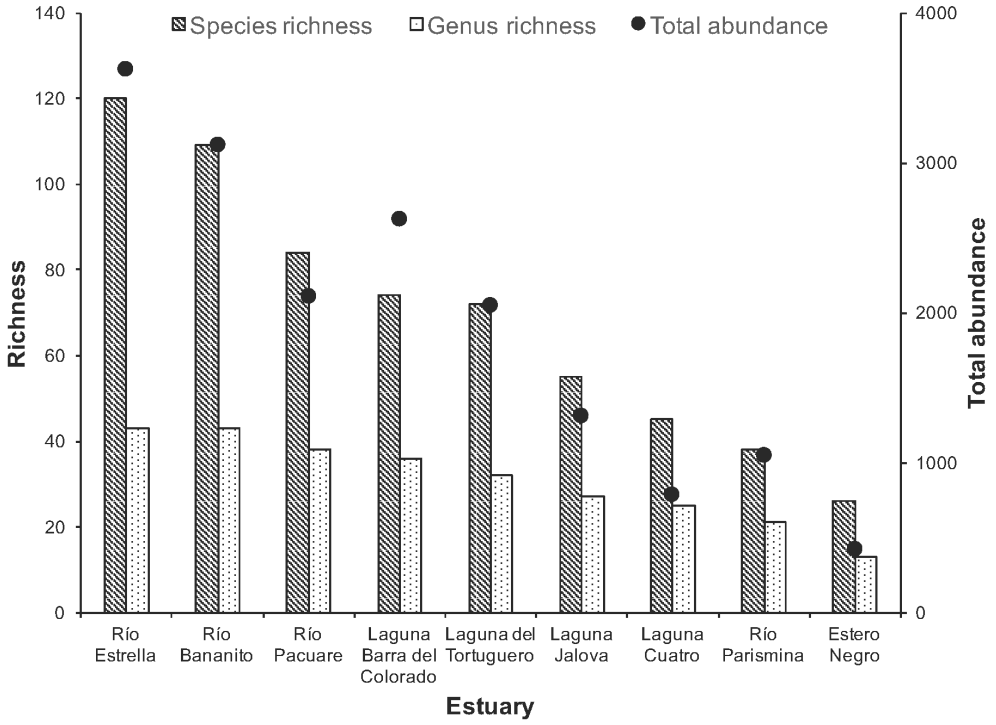

Figure 3.2: Species and genus richness and total abundance of pupal exuviae samples from nine estuaries.

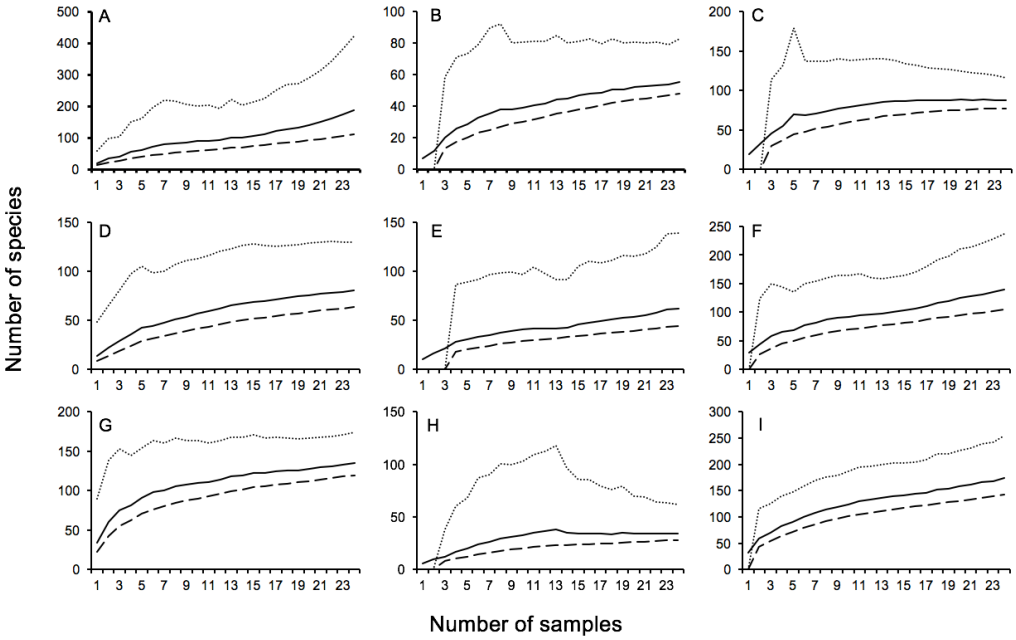

Figure 3.3: Species accumulation curves for Laguna Barra del Colorado (A), Laguna Cuatro (B), Laguna del Tortuguero (C), Laguna Jalova (D), Río Parismina (E), Río Pacuare (F), Río Bananito (G), Estero Negro (H), and Río Estrella (I). Solid black line indicates mean Chao 1 species-richness estimator, and dotted and dashed black lines indicate Chao 1 upper and lower 95% confidence level, respectively.

Table 3.1: Costa Rica study watersheds, estuaries, GPS coordinates, percent forest land cover, and average physiochemical parameters of the water.

Watershed	Estuary	Coordinates	% Forest	Water temp.	DO	Salinity	Secchi
Chirripo	Laguna Barra del Colorado	10.7650 N, -83.4920 W	73.3	26.4	6.3	0.1	0.3
Tortuguero	Laguna Cuatro	10.6330 N, -83.5458 W	53.7	26.4	1.7	7.0	1.1
Tortuguero	Laguna del Tortuguero	10.5586 N, -83.5127 W	53.7	26.8	4.7	13.0	0.9
Tortuguero	Laguna Jalova	10.3475 N, -83.3952 W	53.7	26.1	5.5	2.0	0.5
Reventazón	Río Parismina	10.3083 N, -83.3554 W	57.9	25.5	7.1	0.1	0.2
Pacuare	Río Pacuare	10.2201 N, -83.2892 W	75.8	25.4	7.0	0.4	0.5
Bananito	Río Bananito	9.8786 N, -82.9622 W	85.4	26.4	6.1	3.9	0.6
Bananito	Estero Negro	9.8459 N, -82.9369 W	85.4	27.5	3.0	9.8	0.5
Estrella	Río Estrella	9.7859 N, -82.8914 W	91.0	24.7	7.2	3.4	0.3

GPS coordinates represent middle zone of estuary sample area, water temp. = average water temperature (°C), DO = average dissolved oxygen (mg/L), average salinity (ppt), Secchi = average Secchi depth (m), % Forest = Percent forest land cover per watershed (data from Kranzfelder et al. (in review-a))

Table 3.2: Tolerance values from Barbour et al. (1999) and Ferrington et al. (2008) used to calculate the biotic index.

Genus	TV	TL	Genus	TV	TL
TANYPODINAE			CHIRONOMINI (Cont)		
Ablabesmyia	6.4	Genus	*Nilothauma*	5.5	Genus
Coelotanypus	6.9	Genus	*Parachironomus*	8.6	Genus
Fittkauimyia	4.6	Tribe	*Paralauterborniella*	4.8	Genus
Labrundinia	5.2	Genus	*Polypedilum*	6.7	Genus
Larsia	8.3	Genus	*Robackia*	3.3	Genus
Monopelopia	6.0	Genus	*Saetheria*	8.1	Genus
Nilotanypus	4.0	Genus	*Stenochironomus*	6.4	Genus
Pentaneura	4.6	Genus	*Stictochironomus*	6.7	Genus
Procladius	9.3	Genus	*Xenochironomus*	7	Genus
Tanypus	9.6	Genus	*Xestochironomus*	6.7	Tribe
Zavrelimyia	9.3	Genus	*Zavreliella*	2.7	Genus
ORTHOCLADIINAE			Chironomini Genus 1	6.7	Tribe
Corynoneura	6.2	Genus	Chironomini Genus 2	6.7	Tribe
Cricotopus	8.7	Genus	Chironomini Genus 3	6.7	Tribe
Eukiefferiella	2.7	Genus	Chironomini Genus 4	6.7	Tribe
Gymnometriocnemus	5	Subfamily	Chironomini Genus 5	6.7	Tribe
Nanocladius	4.9	Genus	Chironomini Genus 6	6.7	Tribe
Onconeura	5	Subfamily	Chironomini Genus 7	6.7	Tribe
Orthocladius	6.5	Genus	Chironomini Genus 8	6.7	Tribe
Orthocladiinae Genus 1	5	Subfamily	Chironomini Genus 9	6.7	Tribe
Orthocladiinae Genus 2	5	Subfamily	Chironomini Genus 10	6.7	Tribe
Orthocladiinae Genus 3	5	Subfamily	Chironomini Genus 11	6.7	Tribe
Parakieffereiella	5.9	Genus	Chironomini Genus 12	6.7	Tribe
Parametriocnemus	4.8	Genus	Chironomini Genus 13	6.7	Tribe
Pseudosmittia	5	Subfamily	PSEUDOCHIRONOMINI		
Stictocladius	5	Subfamily	*Pseudochironomus*	4.2	Genus
Thienemanniella	6	Genus	TANYTARSINI		
Ubatubaneura	5	Subfamily	*Cladotanytarsus*	3.7	Genus
CHIRONOMINI			*Paratanytarsus*	7.7	Genus
Apedilum	6.2	Genus	*Rheotanytarsus*	6.4	Genus
Axarus	6.7	Tribe	*Stempellina*	2	Genus
Beardius	6.7	Tribe	*Stempellinella*	5.3	Genus
Chironomus	9.8	Genus	*Sublettea*	1.7	Genus
Cladopelma	2.5	Genus	*Tanytarsus*	6.7	Genus
Cryptochironomus	6.7	Genus			
Cryptotendipes	6.1	Genus			
Dicrotendipes	9.1	Genus			
Einfeldia	9.8	Genus			
Goeldichironomus	10	Genus			
Harnishia	7.5	Genus			
Microchironomus	6.7	Tribe			

TL indicates the taxonomic level tolerance value that was used for analysis

TV = tolerance value

Table 3.3: Estuary biotic integrity ratings for interpreting overall CIBI scores.

CIBI Score	Biotic Integrity Rating
80-100	Excellent
60-79	Good
40-59	Fair
20-39	Poor
10-19	Very Poor

Table 3.4: Number of genera collected from each subfamily/tribe at nine different estuaries.

Estuary	Tanypodinae	Orthocladiinae	Chironomini	Pseudochironomini	Tanytarsini	Total
Laguna Barra del Colorado	3	8	20	1	4	36
Laguna Cuatro	4	4	15	0	2	25
Laguna del Tortuguero	5	6	15	1	5	32
Laguna Jalova	2	4	16	1	4	27
Río Parismina	1	6	12	0	2	21
Río Pacuare	5	10	19	0	4	38
Río Bananito	6	10	21	1	5	43
Estero Negro	3	4	5	0	1	13
Río Estrella	5	11	21	1	5	43

Table 3.5: Relative abundance (% Orthocladiinae, % Chironomini, and %Tanytarsini) and relative richness (%Tolerant and % Intolerant) values at nine different estuaries.

Estuary	% Orthocladiinae	% Chironomini	% Tanytarsini	% Tolerant	% Intolerant
Laguna Barra del Colorado	58.0	34.7	4.3	11.1	0.0
Laguna Cuatro	2.3	57.2	38.3	28.0	8.0
Laguna del Tortuguero	8.5	13.4	70.1	18.8	9.4
Laguna Jalova	10.5	35.1	49.0	22.2	3.7
Río Parismina	46.8	46.5	4.6	9.5	9.5
Río Pacuare	26.5	64.2	8.0	13.2	5.3
Río Bananito	21.9	39.5	33.3	20.9	2.3
Estero Negro	2.1	72.6	12.8	23.1	0.0
Río Estrella	27.0	13.4	29.0	14.0	7.0

Table 3.6: Richness (Total Species Richness & Margalef's Index), relative abundance (Berger-Parker Index), biotic index (Hilsenhoff's Biotic Index), and diversity index (Shannon's Index) values at nine different estuaries.

Estuary	Total Species Richness	Berger-Parker Index	Shannon's Index	Margalef's Index	Hilsenhoff's Biotic Index
Laguna Barra del Colorado	74	0.384	2.38	9.27	5.74
Laguna Cuatro	45	0.236	2.67	6.60	6.33
Laguna del Tortuguero	72	0.636	1.89	9.31	6.63
Laguna Jalova	55	0.440	2.22	7.52	5.17
Río Parismina	38	0.420	2.00	5.32	5.92
Río Pacuare	84	0.177	3.04	10.85	6.23
Río Bananito	109	0.150	3.38	13.43	6.10
Estero Negro	26	0.563	1.71	4.13	6.62
Río Estrella	120	0.136	3.37	14.52	6.36

Table 3.7: Metric scores for each estuary.

Estuary	% Orthocladiinae	% Chironomini	% Tanytarsini	% Tolerant	% Intolerant
Laguna Barra del Colorado	10	7	1	10	1
Laguna Cuatro	1	3	6	1	8
Laguna del Tortuguero	1	10	10	6	9
Laguna Jalova	2	7	7	4	4
Río Parismina	8	5	1	10	10
Río Pacuare	5	2	1	9	6
Río Bananito	4	6	5	4	2
Estero Negro	1	1	2	3	1
Río Estrella	5	6	4	8	7

Table 3.8: Metric scores and Chironomidae Index of Biotic Integrity (CIBI) scores.

Estuary	Total Species Richness	Berger-Parker Index	Shannon's Index	Margalef's Index	Hilsenhoff's Biotic Index	CIBI score
Laguna Barra del Colorado	6	6	4	5	7	57
Laguna Cuatro	3	9	6	3	3	43
Laguna del Tortuguero	5	1	2	5	1	50
Laguna Jalova	4	4	3	4	10	49
Río Parismina	2	5	2	2	6	51
Río Pacuare	7	10	8	7	4	59
Río Bananito	9	10	10	9	5	64
Estero Negro	1	2	1	1	1	14
Río Estrella	10	10	10	10	3	73

CHAPTER 4: Trace DNA from insect skins: a comparison of five extraction protocols and direct PCR on chironomid pupal exuviae

Summary

Insect skins (exuviae) are of extracellular origin and shed during molting. The skins do not contain cells or DNA themselves, but epithelial cells, hairs and other cell-based structures might accidentally attach as they are shed. This source of trace DNA can be sufficient for PCR amplification and sequencing of target genes and aid in species identification through DNA barcoding or association of unknown life stages. However, it requires a DNA isolation protocol that optimizes the output of target DNA. Here we compare the relative effectiveness of five different DNA extraction protocols and direct PCR in isolation of DNA from chironomid pupal exuviae. Chironomidae (Insecta: Diptera) is a species-rich group of aquatic macroinvertebrates widely distributed in freshwater environments and considered a valuable bioindicator of water quality. Genomic DNA was extracted from 61.2% of 570 sampled pupal exuviae. There were significant differences in the methods with regards to cost, handling time, DNA quantity, PCR success, sequence success, and the ability to sequence target taxa. The NucleoSpin® Tissue XS Kit, DNeasy® Blood and Tissue kit, and QuickExtract™ DNA Extraction Solution provided the best results in isolating DNA from single pupal exuviae. Direct PCR and DTAB/CTAB methods gave poor results. While the observed differences in DNA isolation methods on trace DNA will be relevant to research that focuses on aquatic macroinvertebrate ecology, taxonomy and systematics, they should also be of interest for

studies using environmental barcoding and metabarcoding of aquatic environments.

Introduction

The effectiveness of DNA barcoding, metabarcoding, and environmental barcoding as a routine practice in biodiversity monitoring and conservation is strongly dependent on the quality of the DNA extractions (Ivanova et al. 2006; Knebelsberger and Stöger 2012). There are a variety of methods to isolate DNA from biological materials, but the ideal extraction technique should provide high DNA quality and quantity, as well as, be efficient in terms of cost and handling time, be suitable for high sample throughput, and generate minimal hazardous waste (Chen et al. 2010). Traditional extraction methods, including phenol/chloroform (Nishiguchi et al. 2002) or DTAB-CTAB lysis (Phillips and Simon 1995), work efficiently for extracting DNA from samples without destruction of museum specimens, but use toxic chemicals and are time-consuming (Hajibabaei et al. 2005). Various commercially available DNA extraction kits, especially silica-membrane based methods, are becoming increasingly popular because of their ease of use for a wide range of biological samples, limited labor, and ability to consistently produce high-quality DNA (Hajibabaei et al. 2005; Nishiguchi et al. 2002). However, some of the drawbacks of these kits include the expense and long incubation times (Ball and Armstrong 2008). Another rapid and low cost DNA barcoding technique termed "direct PCR" facilitates PCR amplification of target genes directly from samples without DNA extraction and purification; however, PCR success rates can be low for many taxa (Wong et al. 2014). Although these standardized DNA isolation methods can be used for

a broad range of biological samples, some are still problematic for low yield or degraded samples (Goldberg et al. 2015; Zimmermann et al. 2008).

Chironomidae (Insecta: Diptera) are a species-rich group of aquatic macroinvertebrates that are abundant and widely distributed in aquatic systems (Cranston 1995). Chironomid communities are considered valuable bioindicators of water quality due to their high species diversity, ubiquity, and varying species sensitivity to environmental disturbances (Ferrington et al. 2008; Marziali et al. 2010; Raunio et al. 2011). A commonly used form of sampling chironomids involves collections of pupal exuviae (Calle-Martínez and Casas 2006; Raunio et al. 2007; Wilson and Ruse 2005), which is the exoskeleton shed by the adult as it emerges from the pupae on the surface of the water (Ferrington et al. 1991) (Fig. 4.1). Pupal exuviae accumulate behind obstructions, like fallen trees, or along banks through the action of wind or water current and can be easily collected to give a comprehensive sample of chironomid communities (Ruse 2010). This collection method has several advantages over traditional benthic larvae sampling techniques: 1) applicability in most aquatic environments including streams and rivers, estuaries, lakes, ponds, pools, and wetlands, 2) accumulated exuviae represent taxa that have originated from a wide range of microhabitats, and 3) sampling and sample processing are easy and rapid (Kranzfelder et al. 2015; Wilson and Ruse 2005).

Species identification is essential for large-scale aquatic bioassessment studies (Jones 2008; Lenat and Resh 2001; Marshall et al. 2006; Melo 2005). However, in many regions, it is difficult to identify chironomid pupal exuviae to species morphologically

since most species descriptions and keys are based on adult males (Carew et al. 2007; Ekrem et al. 2007). Ekrem et al. (2007), among others, have found that partial mitochondrial gene cytochrome oxidase I (COI) gene sequences can be used to link different life stages of Chironomidae and DNA barcoding has been successful for identifying cryptic and undescribed chironomid species from whole specimens of larvae, pupae, and adults (Anderson et al. 2013a; Brodin et al. 2013; Meier et al. 2015; Stur and Ekrem 2011). Yet, barcoding of chironomid pupal exuviae has been challenging due to the low quantities of genomic DNA available for extraction (Krosch and Cranston 2012). The exoskeleton of the pupal exuviae is made of extracellular chitin, the most widespread structural polysaccharide in nature (Merzendorfer 2006), and does not contain any nucleic acids. Therefore, genomic DNA yield from pupal exuviae relies on muscle tissue, hairs, and epithelial cells lining the foregut, hindgut, and tracheae that are left behind on the inner surface of the cuticle by the emerging adult. This source of trace DNA cab be sufficient for PCR amplification and sequencing of target genes (Krosch and Cranston 2012). Miller et al. (1988) attempted to extract DNA from chironomid pupal exuviae using a modified salting out protocol, but had no success with this method. Recently, Krosch and Cranston (2012) were able to extract genomic DNA from 27 of 58 chironomid pupal exuviae using one extraction method (DNeasy® Blood & Tissue Kit), but this study was limited by few specimens, one extraction method, and lack of quantitative data. Consequently, there is no consensus regarding the most effective DNA isolation method for chironomid pupal exuviae or similar trace DNA samples.

In this paper, we compare the performance of five different DNA extraction methods and direct PCR in isolation of genomic DNA from chironomid pupal exuviae in terms of cost, handling time, DNA quantity, PCR success, sequence success, and ability to sequence target taxa.

Materials and Methods

Sample collection and preparation

Chironomidae pupal exuviae samples were collected using drift nets from lake Lianvatnet (N 63.403°, E 10.318°) and Nidelva River (N 63.429°, E 10.379°) both in Trondheim, Sør-Trøndelag, Norway between August and October 2014. Pupal exuviae were picked from the nets and/or larval trays using un-sterilized forceps and put into absolute ethanol (>99.8%). Individual pupal exuviae were identified under a dissecting microscope, separated into sterile 1.5 mL microcentrifuge tubes filled with absolute ethanol, and stored in dark conditions at 2°C for up to four months. Before extraction, pupal exuviae were air dried on a piece of paper and transferred into sterile 1.5 microcentrifuge tubes. This was done in the pre-PCR room of a standard molecular lab.

DNA extraction

For each of the five extraction protocols, total genomic DNA was isolated from 95 pupal exuviae belonging to 13 different taxonomic groups (Table 4.1). Each method had one negative control. The five DNA extraction methods included: a dodecyltrimethylammonium bromide/cetyltrimethylammonium bromide (DTAB/CTAB) protocol adapted from (Nishiguchi et al. 2002), three commercially-available kits:

DNeasy® Blood & Tissue Kit (Qiagen, Hilden, Germany), NucleoSpin® Tissue XS Kit (Macherey-Nagel, Düren, Germany), E.Z.N.A.® Insect DNA Kit (Omega Bio-Tek Inc., Norcross, Georgia, USA), and an extraction solution: QuickExtract™ DNA Extraction Solution (Epicentre, Eindhoven, Netherlands).

For the DTAB/CTAB lysis protocol, DNA was extracted according to protocol outlined by (Nishiguchi et al. 2002) with the following modifications: (a) Tissue was digested at 68°C in 600 µL of DTAB solution overnight. (b) After digestion, pupal exuviae were removed carefully with a plastic pipette tip and transferred to absolute ethanol. (c) Pellets were washed in 300 µL of 70% ethanol as suggested by (Phillips and Simon 1995). (d) Pellets were resuspended in 20 µL of TE buffer.

For the three commercially available kits, DNA was extracted according to the manufacturer's instructions with the following modifications:

1. DNeasy® Blood & Tissue Kit: DNA was extracted similar to (Krosch and Cranston 2012). (a) Tissue was digested with proteinase K overnight at 37°C; (b) After digestion, pupal exuviae were removed carefully with a plastic pipette tip and transferred to absolute ethanol; (c) To maximize DNA yield, two successive elution steps (first elution: 70 µl, second elution: 30 µl), were performed for a final elution volume of 100 µl; (d) Incubation time of elution was increased to 5 minutes.

2. NucleoSpin® Tissue XS Kit: (a) Tissue was digested with Buffer T1 and proteinase K overnight at 37 °C; (b) After digestion, pupal exuviae were removed carefully with a plastic pipette tip and transferred to absolute ethanol; (c) Incubation time

of elution was increased to 5 minutes; (d) Elution fraction was incubated with open lid for 17 minutes at 75°C.

3. E.Z.N.A.® Insect DNA Kit: (a) Tissue was digested with CTL Buffer and proteinase K overnight at 37°C; (b) After digestion, pupal exuviae were removed carefully with a plastic pipette tip and transferred to absolute ethanol; (c) To maximize DNA yield, two successive elution steps (first elution: 70 µl, second elution: 30 µl), were performed for a final elution volume of 100 µl; (d) Incubation time of elution was increased to 5 minutes.

For the extraction solution, QuickExtract™ DNA Extraction Solution, DNA was extracted according to the manufacturer's instructions with the following modification: tissue was digested with 100 µL of QuickExtract™ DNA Extraction Solution.

During data analysis, DNA extracts were stored at −20°C. After analysis, DNA extracts were moved to long-term storage at −80°C. All reference material (DNA extracts & voucher specimens) for this study is deposited at the NTNU University Museum, Norwegian University of Science and Technology, Trondheim, Norway.

DNA quantity was measured with a Qubit® double-stranded DNA (dsDNA) High Sensitivity (HS) Assay Kit (Life Technologies, Eugene, OR, USA) and read a by Qubit® 2.0 Fluorometer (Life Technologies, Eugene, OR, USA). For the DTAB/CTAB method, DNeasy® kit, E.Z.N.A.® kit, and QuickExtract™ method 14 µL of extracted DNA template were used in 186 µL of the dsDNA HS assay. For the NucleoSpin® kit, 8 µL of extracted DNA template were used in 192 µL of the dsDNA HS assay. The Qubit® 2.0 Fluorometer calculates DNA concentration based on the fluorescence of dye that only

fluoresce when bound to dsDNA. The fluorometer picks up this fluorescence signal and converts it into a DNA concentration measurement using DNA standards of known concentration. DNA quantity was calculated with a simple multiplication based on DNA concentration derived from the Qubit measurements and the volume of the DNA extract. Samples with DNA concentrations of less than 0.0005ng/ μL were too low to be read by the Qubit flurometer and were counted as zero values for data analysis.

DNA extraction cost, handling time, and user friendliness

The cost per sample for each extraction method was estimated based on the price of chemicals and enzymes consumed. The handling time per sample was calculated as the time required starting from dehydration of the pupal exuviae to DNA extract ready for PCR. If required in the method, then time included the 15-hour overnight incubation, but not the time spent preparing solutions for the DTAB/CTAB method. User friendliness was described with advantages and disadvantages of labor associated with DNA isolation method as observed by the first author.

PCR optimization, amplification, and purification

PCR amplification was optimized by initially testing two DNA polymerases, HotStarTaq (Qiagen, Hilden, Germany) and *TaKaRa Ex Taq® Hot Start (*Takara Bio Inc., Otsu, Shiga, Japan), and two thermocycling programs (Appendix G) on seven samples plus one negative control per DNA polymerase and thermocycling program combination. The combination of the TaKaRa Taq and second thermocycling program

produced strong, narrow bands and was used for all samples in this study. For the five DNA extraction methods, a mixture of 2 µL of DNA template and 23 µL of master mix (15.8 µL of DNase-free, sterile water, 2.5 µL of 10X Ex Taq Buffer, 1 µL of 10 µM forward and reserve primer, 2.0 µL of 2.5 mM dNTP mixture, 0.5 µL of 20 mM $MgCl_2$, and 0.2 µL of *TaKaRa Ex Taq® Hot Start*) was prepared for each sample. For the direct PCR method (adapted from (Wong et al. 2014)), 23 µL of the same master mix was prepared and pipetted into wells containing the 95 dried pupal exuviae. For all methods, PCR amplification on the mitochondrial cytochrome c oxidase subunit I (COI) gene was carried out with primer pair LCO1490/HCO2198 (Folmer et al. 1994) using a BioRad C1000™ Thermal Cycler. PCR amplification results for all methods were checked with electrophoresis on 1% agarose gel stained with SYBR® Safe DNA gel stain (Life Technologies, Carlsbad, CA, USA). Successfully amplified PCR products were cleaned with Illustra™ ExoProStar 1-Step (GE Healthcare Life Sciences, Little Chalfont, UK) following the manufacturer's instructions. PCR success was indicated by the presence of a band for each sample on an agarose gel.

Sanger sequencing

Purified PCR products were sequenced in the forward direction (5'→3'); one-directional sequencing can yield >500 bases, which is sufficient for routine species delimitation (Wong et al. 2014). The Sanger sequencing was done at Eurofins Genomics (Ebersberg, Germany). Sequences were trimmed using Sequencher version 4.8 (Gene Codes Corp., Ann Arbor, Michigan, USA). PCR primers were trimmed from read ends,

as necessary. Nucleotide sequence similarities were blasted against the BOLD (Barcode of Life Data Systems, http://www.boldsystems.org/) Identification Engine using the full database. When the BOLD Identification Engine failed to find a match, then the sequences were blasted against GenBank (http://www.ncbi.nlm.nih.gov/genbank/). The FASTA files generated in this study with high quality trace files matching morphologically identified target taxa were uploaded to BOLD and are made publicly available under the project name "Trondheim Chironomidae Pupal Exuviae (TRPEX)." Other raw data is available from the first author upon request. Sequence success was indicated by the presence of a high-quality sequence (quality score of 80% or above in Sequencher version 4.8) for each sample.

Statistical analyses

To test the likelihood that handling time varies between DNA extraction methods and direct PCR, a Kruskal-Wallis test was combined with a pairwise multiple comparison using Tukey-Kramer (Nemenyi) test (Pohlert 2015). To test the likelihood that DNA quantity (log (x+1) transformed) varies between DNA extraction methods, a one-way analysis of variance (ANOVA) was combined with a pairwise multiple comparison using Tukey's test. To test the likelihood that PCR success, sequence success, and target sequence success varies between DNA extraction methods and direct PCR, binomial generalized linear models (GLMs) were combined with a pairwise multiple comparison using Tukey's test (Hothorn et al. 2008). The pairwise comparisons are based on odds ratios. If the 95% confidence interval of the odds ratio includes the number one, then

there is no significant difference. If the confidence interval of the odds ratio does not include the number one, then there is a significant difference (Szumilas 2010). The DTAB/CTAB method had no PCR success, sequence success, or target sequence success, and was thus excluded from the GLMs and Tukey's tests. For all tests, the statistical significance level was set at 0.05 and was computed in R version 3.1.3 (R Development Core Team 2016). Descriptive statistics are displayed in Table 4.3.

Results

DNA extraction cost, user friendliness, and handling time

The direct PCR was the cheapest method with no associated extraction cost followed closely by the QuickExtract™ method and DTAB/CTAB method. On the other hand, the three commercial kits: NucleoSpin® kit, E.Z.N.A.® kit, and DNeasy® kit, had an increased cost per sample by a factor of approximately six. With regards to user-friendliness, the QuickExtract™ method was the easiest method to use due to easy-to-follow, rapid vortex and incubation procedures and lack of hazardous chemicals. The DTAB/CTAB method was the most technically difficult to perform and required the use of toxic chemicals, such as chloroform. All other methods had about the same moderate labor intensity (Table 4.2).

Handling time per sample was significantly different between the five DNA extraction methods and direct PCR ($\chi^2(5) = 531.586$, $P < 0.001$). The most rapid DNA isolation method was the direct PCR method ($\overline{x} = 1.90$ minutes) followed by the next most time-efficient method, the QuickExtract™ method ($\overline{x} = 4.45$ minutes), which were

statistically different from each other ($P < 0.001$) and all other methods ($P < 0.001$). The most time-consuming methods, E.Z.N.A.® kit ($\overline{x}$ = 48.04 minutes) and the DTAB/CTAB method ($\overline{x}$ = 47.1 minutes), were statistically similar ($P > 0.05$), but statistically different when compared to the statistically similar ($P > 0.05$) DNeasy® kit ($\overline{x}$ = 44.65 minutes) and NucleoSpin® kit ($\overline{x}$ = 44.80) (Table 4.3).

DNA quantity

Genomic DNA was extracted from 349 of 570 sampled pupal exuviae (61.2%). DNA quantity was significantly different between the five DNA extraction methods ($F(4) = 6.234$, $p < 0.001$). The highest DNA quantity resulted from the QuickExtract™ method ($\overline{x}$ = 0.020 ng/μL) this was statistically similar ($P > 0.05$) to the NucleoSpin® kit ($\overline{x}$ = 0.019 ng/μL) and the DNeasy® kit ($\overline{x}$ = 0.010 ng/μL). The E.Z.N.A.® kit ($\overline{x}$ = 0.003 ng/μL) and the DTAB/CTAB method ($\overline{x}$ = 0.004 ng/μL) had statistically similar ($P > 0.05$) low DNA quantity and were statistically different ($P < 0.05$) compared to the QuickExtract™ method and NucleoSpin® kit, but not the DNeasy® kit ($P > 0.05$) (Table 4.3 & Fig. 4.2).

PCR success

PCR amplification success was significantly different between the four DNA extraction methods and direct PCR ($P < 0.001$). The DTAB/CTAB method resulted in no PCR success (Fig. 4.2). The NucleoSpin® kit ($\overline{x}$ = 98.9%) and DNeasy® kit ($\overline{x}$ = 97.9%) were statistically similar ($P > 0.05$) and had good PCR success. The E.Z.N.A.® kit ($\overline{x}$ = 83.2%) and QuickExtract™ method ($\overline{x}$ = 83.2%) were statistically similar ($P > 0.05$) and

had moderate PCR success, but were statistically differed from the NucleoSpin® kit and the DNeasy® kit ($P < 0.05$). Direct PCR ($\bar{x} = 2.1\%$) had very low PCR success and was statistically different from all other methods ($P < 0.001$) (Table 4.4 & Fig. 4.3).

Sequence success

High-quality sequences were obtained from 126 of the 349 sequenced pupal exuviae (36.1%). Sequence success was significantly different between the four DNA extraction methods and direct PCR ($P < 0.001$). The DTAB/CTAB method resulted in no sequence success (Fig. 4.2). The NucleoSpin® kit ($\bar{x} = 38.9\%$), the QuickExtract™ method ($\bar{x} = 36.8\%$), and the DNeasy® kit ($\bar{x} = 28.4\%$) were statistically similar ($P > 0.05$) and resulted in the highest sequence success. The E.Z.N.A.® kit ($\bar{x} = 17.9\%$) produced statistically lower sequence success than the NucleoSpin® kit and QuickExtract™ method ($P < 0.05$). The direct PCR ($\bar{x} = 2.1\%$) had statistically lower sequencing success than all other methods ($P < 0.001$) (Table 4.4 & Fig. 4.3).

Target sequence success

High-quality sequences matching chironomid taxonomic groups were obtained from 48 of the 349 sequenced pupal exuviae (13.7%). Out of all of the high-quality sequences, 39.1% matched chironomid sequences, while 60.9% matched non-target taxa including: cladocerans, *Holopedium gibberum* (43.1%), humans (13.8%), water molds, *Aphanomyces* spp. (1.6%), copepods, *Drepanopus* spp. (0.8%), a sea slug, *Flabellina verrucosa* (0.8%), and a brown hydra, *Hydra oligactis* (0.8%). Target taxa sequence

success was significantly different between the five DNA extraction methods and direct PCR ($P < 0.001$). The DTAB/CTAB method resulted in no target sequence success. The DNeasy® kit ($\bar{x}$ =17.9%), NucleoSpin® kit ($\bar{x}$ = 14.7%), and QuickExtract™ method ($\bar{x}$ = 12.6%) resulted in highest ability to sequence target taxa and were statistically similar ($P > 0.05$). The E.Z.N.A.® kit ($\bar{x}$ = 4.21%) and direct PCR ($\bar{x}$ = 1.1%) had significantly lower sequence success than the DNeasy® kit ($P < 0.05$), but performed similar to the QuickExtract™ method and NucleoSpin® kit ($P > 0.05$) (Table 4.4 & Fig. 4.3).

Discussion

There were significant differences in the five DNA extraction methods and direct PCR with regards to cost, handling time, DNA quantity, PCR success, sequence success, and the ability to sequence target taxa. Considering all criteria described in the present study, three methods: the NucleoSpin® Tissue XS Kit, the DNeasy® Blood and Tissue kit, and the QuickExtract™ DNA Extraction Solution, provided the best results in isolating trace DNA from single chironomid pupal exuviae. These three DNA extraction methods gave the highest DNA quantity, PCR success, sequence success, and ability to sequence target taxa and there were no significant differences between these methods with regards to these factors, except PCR success. High PCR success is especially important for molecular studies that plan to extract DNA from only a small number of highly valuable specimens (e.g. specimens collected under hazardous conditions or in areas difficult to access); and therefore, we suggest the NucleoSpin® Tissue XS Kit and the DNeasy® Blood and Tissue kit for these types of sensitive molecular studies. The

NucleoSpin® Tissue XS Kit has specialized columns (small diameter and funnel-shaped) that are designed for small volumes of elution buffer to be dispensed accurately, thereby, increasing the final DNA concentration (Macherey-Nagel 2015). However, in our study the NucleoSpin® kit did not perform significantly better in terms of DNA yield or PCR success compared to the DNeasy® Blood and Tissue kit. A disadvantage of the NucleoSpin® kit is the recommended final elution volume of 20 µL compared to 100 µL for the other commercial kits. Thus, reducing the amount of DNA extract available for downstream analyses, DNA quantification, and DNA banking. The DNeasy® Blood & Tissue Kit is a standardized method for a variety of sample types designed for high DNA yields and quantity (Qiagen 2015). It has successfully been used to extract DNA from chironomid pupal exuviae (Krosch and Cranston 2012), larvae (Cao et al. 2013; Kelley et al. 2014), and adults (Carew et al. 2013; Ekrem et al. 2007). Subsequently, it is perhaps not surprising that this kit was efficient for DNA isolation of trace DNA in chironomid pupal exuviae. The success of these commercial kits is consistent with that of Hajibabaei et al. (2005) who compared five DNA extraction methods and found that silica-membrane binding methods were the most effective. Likewise, results of Zetzsche et al. (2008) and Dittrich-Schroder et al. (2012) suggest that silica-membrane methods provide the best results for routine isolation of high quality genomic DNA.

While the NucleoSpin® Tissue XS Kit and DNeasy® Blood and Tissue kit resulted in significantly higher PCR success, the QuickExtract™ DNA Extraction Solution was significantly more time-efficient. In previous studies, this method successfully extracted DNA from other aquatic invertebrates including: freshwater

mussels (Unionidae) (Christian et al. 2007), freshwater worms (*Rhyacodrilus falciformis)* (Martinsson et al. 2013), and cladocerans (*Daphnia* spp.) (Vergilino et al. 2009). The very low cost and handling time per sample, as well as, the lack of toxic chemicals makes QuickExtract™ the most time- and cost-efficient approach among the best performing methods tested. Cost and handling time might be of importance for studies requiring high volume DNA extraction and for instance rapid, cost-effective assembly of DNA barcodes. We would consider this extraction method for molecular studies that have a large sample sizes and/or are limited in funding.

The E.Z.N.A.® Insect DNA Kit performed similar to the QuickExtract™ DNA Extraction Solution with regards to PCR success, but performed lower for all other variables. The kit is designed to extract DNA from 20 mg insect or related arthropod tissue (Omega Bio-Tek Inc. 2015), which is much higher than the residual tissue present within pupal exuviae. Additionally, the use of toxic chemicals, such as chloroform, and many vortex and incubation steps make this kit comparatively unfriendly to users. We therefore do not favor this kit as one of the top choices for isolating trace DNA.

Two of the tested DNA isolation methods, DTAB/CTAB and direct PCR, appear to be unsuccessful at recovering/amplifying genomic DNA from chironomid pupal exuviae. The DTAB/CTAB method has been used extensively to extract DNA from small quantities of a wide variety of arthropod museum specimens (Nishiguchi et al. 2002; Phillips and Simon 1995) and was included for comparative purposes. DTAB and CTAB are detergents that form insoluble complexes with nucleic acids, leaving carbohydrates, protein, and many other contaminants in solution. The insoluble precipitate is collected

by centrifugation and re-suspended in a salt solution, which causes the complex to break down, releasing the purified DNA (Giles and Brown 2008). However, the lack of COI gene positive-PCR products suggests that the DTAB/CTAB method is not effective in isolating DNA from chironomid pupal exuviae. Direct PCR has been successfully optimized for a small range of invertebrates for partial COI sequences: Chironomidae, Culicidae, Drosophilidae, Dolichopodidae, Sepsidae (all Dipera); Oreasteridae (Asteroidea) (Wong et al. 2014). Chironomid larvae and adults are particularly convenient sources of tissue for direct PCR (Wong et al. 2014); conversely, pupal exuviae contain a low number of cells that ultimately yield genomic DNA, which could be a reason why this method performed significantly worse than the DNA extraction kits in producing PCR-positive products of COI. Only two pupal exuviae lead to successful sequences, one chironomid and one field-contaminated sequence (water mold, *Aphanomyces)*.

While three of the six extraction methods efficiently extracted DNA from chironomid pupal exuviae, many of the high-quality COI sequences that were retrieved matched non-target taxa and a number of the low-quality sequences clearly showed double peaks in the chromatogram. This suggests that contaminant DNA was amplified instead of or alongside the DNA from the targeted chironomid taxonomic groups. DNA extraction controls and PCR controls were negative; however, some of the good-quality contaminant sequences (e.g. human and sea slug) could be explained by the carrier effect (Handt et al. 1994). Carrier DNA contamination occurs when a low number of contaminating molecules present in the extraction of amplification reagents absorb to

plastic ware, but do not yield positive PCR products. However, when low-quantity DNA extract, such as from our samples, is added, then the contaminating molecules on the plastic surface are displaced by the DNA extract and these contaminants can become available for PCR amplification (Handt et al. 1994). Low-quality or trace DNA samples compete with contaminant free DNA over the course of an experiment (Lusk 2014), but stricter laboratory precautions, such as routine UV irradiation of reagents and tools and positive pressure laboratory ventilation systems, could reduce chances of free DNA contamination from the lab (Champlot et al. 2010; Willerslev and Cooper 2005).

While lab contamination is probable, the majority of the detected cross-contaminations most likely were introduced from the field. The source of the main non-target DNA (*Holopedium gibberum*) is expected to be from the Nidelva River, since moderate densities of this cladoceran were observed to be floating on the surface of the water during fieldwork. Even the contamination with human DNA might have originated from the river, as there is periodical sewage run-off upstream of the sampling locality. During sample sorting, individual pupal exuviae were inspected under a dissecting microscope and discarded if they had evidence of another organism attached. Nonetheless, cells or free DNA from non-target organisms could get stuck inside or attach to different parts of the pupal exuviae. It is therefore important to consider natural contamination when designing a sampling regime for molecular studies on chironomid pupal exuviae or other trace DNA objects, even if it requires an increase in sampling effort and laboratory processing. Additionally, forceps can be soaked in 50% bleach solution, rinsed in water, and dried between samples to reduce cross-contamination

(Goldberg et al. 2013).

Chironomid pupal exuviae are among the most useful and cost-efficient life stages to include in aquatic biodiversity studies (Ferrington and Coffman 2014; Kranzfelder and Ferrington Jr. 2015; Raunio et al. 2011), but morphological species identification is challenging due to lack of species associations and comprehensive taxonomic keys. The association of chironomid pupal exuviae with adult counterparts using DNA barcodes can further increase the usefulness of this life stage in freshwater science. The use of an effective protocol for DNA isolation of trace DNA in insect exuviae is crucial and we expect our results to be particularly useful for research focused on ecology, taxonomy and systematics of aquatic macroinvertebrates that shed their exoskeleton during metamorphosis. In addition, our results might be attractive for studies utilizing environmental DNA (eDNA) since high throughput sequencing techniques enables non-target contaminants to be easily filtered out from the resulting sequences.

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Tables and Figures

Table 4.1: Number of pupal exuviae used from each chironomid taxonomic group.

Taxon	*N*
Tanypodinae	30
Prodiamesa olivacea	60
Diamesinae	6
Micropsectra spp.	60
Chironomini	30
Eukiefferiella sp. 1	60
Tvetenia calvescens	60
Eukiefferiella sp. 2	60
Orthocladius spp.	60
Paracricotopus sp.	60
Corynoneurini	30
Tanytarsus spp.	6
Cricotopus sp.	48

N, number of pupal exuviae

Table 4.2: Comparison of cost per sample and user friendliness of five DNA extraction methods and direct PCR.

Method	Cost per sample	User friendliness	
		Advantages	Disadvantages
DNeasy® Blood & Tissue Kit	$3.16	Simple; kit method with all buffers supplied	Overnight incubation; more expensive than non-kit methods
NucleoSpin® Tissue XS Kit	$2.17	Simple; kit method with all buffers supplied; designed for very small samples	Overnight incubation; more expensive than non-kit methods; low final elution volume
E.Z.N.A.® Insect DNA Kit	$2.20	Kit method with all buffers supplied; designed for very small samples	Overnight incubation; more expensive than non-kit methods; use of toxic chemicals; many steps
QuickExtract™ DNA Extraction Solution	$0.45	Inexpensive; simple; rapid (no overnight incubation); non-toxic chemicals	Additional vortex steps could damage voucher specimens
DTAB/CTAB lysis	$0.65	Inexpensive	Overnight incubation; user-made buffers may introduce contamination; use of toxic chemicals, many steps
Direct PCR	$0.00	No extraction cost or time	Slightly increased specimen handling could introduce contamination

Cost per sample is based on 2014 list prices in U.S. dollars. User friendliness is based on first author personal observations.

Table 4.3: Evaluation of relative effectiveness of five DNA extraction methods and direct PCR.

Method	Time (minutes)		DNA quantity (ng/µL)		PCR success (%)		Sequence success (%)		Target sequence success (%)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
DNeasy® Blood & Tissue Kit	44.65	0.36	0.010	0.006	97.89	14.43	28.42	45.34	17.89	38.53
NucleoSpin® Tissue XS Kit	44.80	0.24	0.019	0.056	98.94	10.26	38.95	49.02	14.74	35.64
E.Z.N.A.® Insect DNA Kit	48.04	1.13	0.003	0.006	83.16	37.62	17.89	38.54	4.21	20.19
QuickExtract™ DNA Extraction Solution	4.45	0.26	0.020	0.055	83.15	37.62	36.84	48.49	12.63	33.40
DTAB/CTAB lysis	47.10	0.25	0.000	0.003	0.00	0.00	0.00	0.00	0.00	0.00
Direct PCR	1.90	0.00	N/A	N/A	2.11	14.43	2.11	14.43	1.05	10.26

Table 4.4: Pairwise comparisons of four DNA extraction methods and direct PCR. (A) PCR success (n = 475), (B) sequence success (n = 475), and (C) target sequence success (n = 475).

Pairwise comparisons	OR	95% CI	P value
(A)			
DNeasy® - Direct PCR	2162.46	144.34-32.53e^{3}	<0.001
E.Z.N.A.® - Direct PCR	229.52	29.58-17.82e^{2}	<0.001
NucleoSpin® - Direct PCR	4372.12	160.77-11.93e^{4}	<0.001
QuickExtract - Direct PCR	229.52	29.58-17.82e^{2}	<0.001
E.Z.N.A.® - DNeasy®	0.11	0.01-0.82	<0.05
NucleoSpin® - DNeasy®	2.02	0.07-54.98	NS
QuickExtract - DNeasy®	0.11	0.01-0.82	<0.05
NucleoSpin® - E.Z.N.A.®	19.03	1.17-310.13	<0.05
QuickExtract - E.Z.N.A.®	1.00	0.35-2.82	NS
QuickExtract - NucleoSpin®	0.05	3.20e^{-3}-0.86	<0.05
(B)			
DNeasy® - Direct PCR	18.47	2.46-138.55	<0.001
E.Z.N.A.® - Direct PCR	10.14	1.30-78.79	<0.05
NucleoSpin® - Direct PCR	29.66	4.01-219.63	<0.001
QuickExtract - Direct PCR	27.11	3.66-201.19	<0.001
E.Z.N.A.® - DNeasy®	0.55	0.21-1.41	NS
NucleoSpin® - DNeasy®	1.61	0.70-3.69	NS
QuickExtract - DNeasy®	1.47	0.64-3.39	NS
NucleoSpin® - E.Z.N.A.®	2.93	1.17-7.31	<0.05
QuickExtract - E.Z.N.A.®	2.68	1.07-6.71	<0.05
QuickExtract - NucleoSpin®	0.91	0.41-2.04	NS
(C)			
DNeasy® - Direct PCR	20.49	1.26-332.91	<0.05
E.Z.N.A.® - Direct PCR	4.13	0.20-84.85	NS
NucleoSpin® - Direct PCR	16.25	0.98-268.15	NS
QuickExtract - Direct PCR	13.59	0.81-227.66	NS

E.Z.N.A.® - DNeasy®	0.20	0.04-0.95	<0.05
NucleoSpin® - DNeasy®	0.79	0.28-2.28	NS
QuickExtract - DNeasy®	0.66	0.22-1.98	NS
NucleoSpin® - E.Z.N.A.®	3.93	0.81-18.97	NS
QuickExtract - E.Z.N.A.®	3.29	0.66-16.29	NS
QuickExtract - NucleoSpin®	0.84	0.27-2.60	NS

OR, odds ratio; CI, 95% confidence interval; NS, not statistically significant ($P > 0.05$).

Figure 4.1: Example of Chironomidae pupal exuviae.

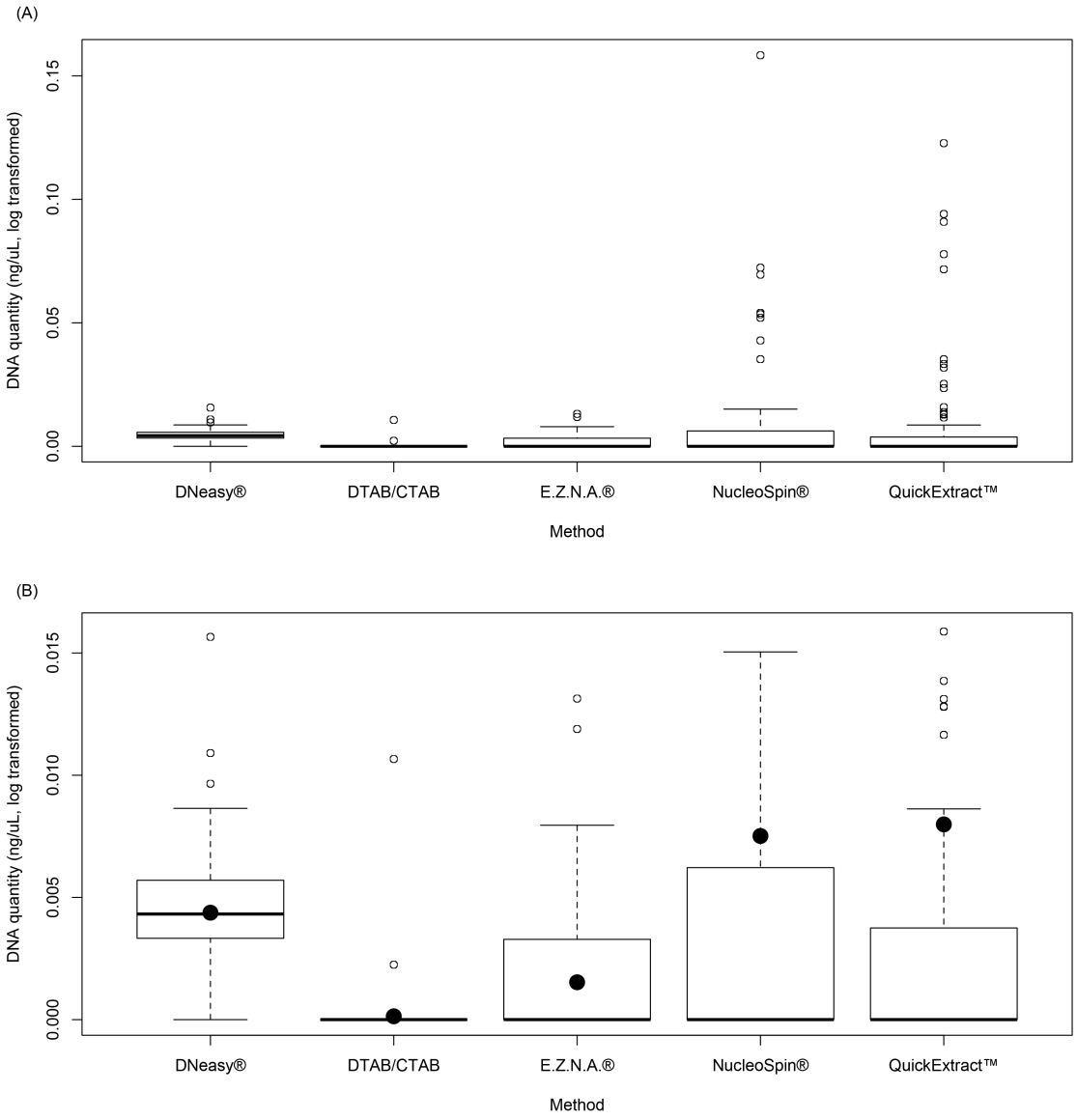

Figure 4.2: Comparison of DNA quantity (ng/μL, log transformed) of five DNA extraction methods. Black lines indicate median, 25th and 75th percentile, whiskers indicate 10th and 90th percentile, open dots indicate outliers, closed dots indicate mean. (A) Data shown with extreme upper outliers. (B) Data shown without extreme upper outliers.

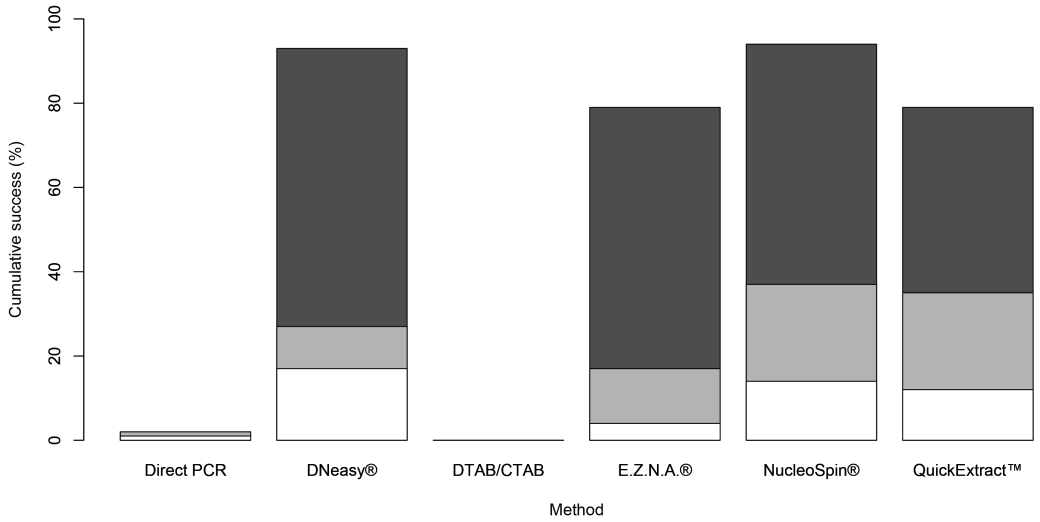

Figure 4.3: Comparison of cumulative percent PCR success, sequence success, and target sequence success for direct PCR and five DNA extraction methods. *Black:* PCR success; *Grey:* Sequence success; *White:* Target sequence success.

CHAPTER 5: DNA barcoding for identification of insect skins: a test on chironomid pupal exuviae

Summary

Chironomidae (Diptera) pupal exuviae samples are commonly used for biological monitoring of aquatic habitats. DNA barcoding has proved useful for species identification of chironomid life stages containing cellular tissue, but the barcoding success of chironomid pupal exuviae is unknown. I assessed whether standard DNA barcoding could be efficiently used for species identification of chironomid pupal exuviae when compared to morphological techniques and if there were differences in performance between temperate and tropical ecosystems, subfamilies, and tribes. PCR, sequence, and identification success differed significantly between geographic regions and taxonomic groups. For Norway, 27 out of 190 (14.2%) of pupal exuviae resulted in high-quality chironomid sequences that match species. For Costa Rica, 69 out of 190 (36.3%) Costa Rican pupal exuviae resulted in high-quality sequences, but none matched known species. Standard DNA barcoding of chironomid pupal exuviae had limited success in species identification of unknown specimens due to contaminations and lack of matching references in available barcode libraries, especially from Costa Rica. Therefore, I recommend future biodiversity studies that focus their efforts on understudied regions, to simultaneously use morphological and molecular identification techniques to identify all life stages of chironomids and populate the barcode reference library with identified sequences.

Introduction

Benthic macroinvertebrates are regularly used for biological monitoring of aquatic habitats, as they are common and widespread, with high species diversity and varying sensitivity to environmental disturbances (Resh 2007; Rosenberg and Resh 1993). Among benthic macroinvertebrates, the family Chironomidae (Diptera), commonly referred to as the non-biting midges or chironomids, is a species-rich aquatic insect group that is particularly sensitive to changes in water quality (Lindegaard 1995; Nicacio and Juen 2015; Pinder 1986). There are close to 1,300 chironomid species recorded from Europe (Sæther and Spies 2013), nearly 900 species described from the Neotropical region (Spies et al. 2009; Spies and Reiss 1996), and estimates that range up to 20,000 species worldwide (Ferrington 2008). Additionally, chironomids are usually the most abundant aquatic insect group in all types of freshwater with larval densities of many thousands per square meter (Anderson et al. 2013b) and also among the most widespread insects inhabiting terrestrial, semi-terrestrial, and aquatic environments in all geographical regions, including Antarctica (Ferrington 2008).

An efficient, low-cost, and easy-to-use method for assessing chironomid communities involves collections of pupal exuviae (Kranzfelder et al. 2015; Raunio and Muotka 2005; Raunio et al. 2007; Wilson and Ruse 2005), which is the exoskeleton shed by the adult as it emerges on the surface of the water. Some advantages offered by collections of pupal exuviae over larval sampling for biological monitoring and assessment are that: (i) deep muddy rivers, canals and lakes, as well as riffles in shallow rivers, may be easily sampled, (ii) a pupal exuviae sample includes taxa from all kinds of

microhabitats, (iii) pupal exuviae collections are more time-efficient in resolving chironomid composition than standard dip-net sampling, and (iv) identification to genus using available keys is relatively easy (Anderson and Ferrington 2012b; Bouchard and Ferrington 2011; Ferrington and Coffman 2014; Wilson and Ruse 2005). A few disadvantages of collections of pupal exuviae over larval sampling are that: (i) emergence is seasonally and diurnally variable, (ii) pupal exuviae float passively on the water's surface, and therefore, it is not possible to know exactly from which area upstream or upwind of the sampling site the pupal exuviae originated, and (iii) traditional morphological species identification requires rearing of adult males from larvae or pupae for life stage association (Wilson and Ruse 2005). These constraints lead to widespread use of generic or higher taxonomic resolution for bioassessments with chironomids, which is problematic since species within a genus or family can display a broad range of sensitivities to various environmental stresses (Sweeney et al. 2011).

DNA barcoding provides an alternative tool for species identification based on a short DNA sequence from a standardized genetic locus (Hebert et al. 2003). A partial region (658 bp) of the cytochrome c oxidase I (COI) gene has been useful for separating cryptic, small, or rare species (Anderson et al. 2013a; Hebert et al. 2004; Jackson et al. 2014; Pauls et al. 2010; Sinclair and Gresens 2008; Stur and Borkent 2014; Sweeney et al. 2011) and associating multiple life stages (Carew et al. 2005; Ekrem et al. 2007; Stur and Ekrem 2011; Webb et al. 2012; Zhou et al. 2009; Zhou et al. 2007). Life stage associations with molecular tools can be particularly valuable for chironomids, since the immature life stages (larvae and pupae) are difficult to separate morphologically to

species, and can be difficult to rear, especially in Neotropical settings with relatively high natural water temperatures (Spies et al. 2009). Carew et al. (2005), Ekrem et al. (2007), Ekrem et al. (2010), and Anderson et al. (2013a) have found that DNA barcodes can be used to link different life stages of the same chironomid. Krosch and Cranston (2012) successfully sequenced 27 out of 58 chironomid pupal exuviae. And more recently, Kranzfelder et al. (2016) successfully isolated genomic DNA from 61.2% of 570 sampled chironomid pupal exuviae. We determined that three DNA extraction kits, the NucleoSpin® Tissue XS Kit, the DNeasy® Blood and Tissue kit, and the QuickExtract™ DNA Extraction Solution, provided the best results in isolating DNA from single pupal exuviae (Kranzfelder et al. 2016). However, we did not study the success of chironomid pupal exuviae species delimitation using DNA barcodes, which is an important precursor for using DNA barcoding as part of bioassessment studies.

The aim of this present study was to assess whether standard DNA barcoding could be efficiently used for species identification of chironomid pupal exuviae when compared to morphological techniques. In addition, I wanted to identify factors that are significantly associated with barcode identification success by investigating if geographic regions (temperate vs. tropical) and taxonomic groups (subfamily and tribe) impact PCR amplification, COI sequencing, and species identification success.

Materials and Methods

Chironomid pupal exuviae were collected using drift nets (mesh size 250 μm) from multiple lentic and lotic aquatic systems in Norway and Costa Rica between August

and December 2014 (Table 5.1). DNA extractions and polymerase chain reaction (PCR) amplifications were done on a total of 380 chironomid pupal exuviae at the NTNU University Museum. Based on results of Kranzfelder et al. (2016), DNA was extracted from 95 individuals using the DNeasy® Blood & Tissue Kit (Qiagen, Hilden, Germany) and from 95 individuals using the QuickExtract™ DNA Extraction Solution (Epicentre, Eindhoven, Netherlands) for a total of 190 individuals from both the Norway and Costa Rica material. See Kranzfelder et al. (2016) for a detailed description of the chironomid pupal exuviae sample collection and preservation methodology and DNA extraction, PCR amplification, and sequencing methodology.

After DNA extraction, voucher specimens were dissected under a stereo microscope and slide mounted in Euparal on individual microscope slides. Finally, slide-mounted specimens were identified under a compound scope to morphospecies or the lowest taxonomic level possible using works by Brundin (1949), Fittkau (1962); Sæther (1981), Epler (1988b); Wiederholm (1986); Wülker (1991), Langton (1991), Jacobsen (2008), Anderson et al. (2013a); Wiedenburg et al. (2009). Species that did not match Linnean names were given interim names (Table 5.2). Subfamilies and tribes were defined based on the results of Cranston et al. (2012). Voucher specimens for the Norway material are deposited at the NTNU University Museum Insect Collection (NTNU-VM). Voucher specimens for the Costa Rica material are deposited at the University of Minnesota Insect Collection (UMSP).

DNA sequences were manually edited in Sequencher version 4.8 (Gene Codes Corp., Ann Arbor, Michigan, USA), checked for stop-codons and frame-shifts, and

aligned by their amino acids using default MUSCLE options (Edgar 2004) in MEGA 6 (Tamura et al. 2013). Ambiguous base calls were given the appropriate International Union of Biochemistry ambiguity symbol. After trimming of uncertain bases at both ends, the aligned sequences were 616 to 630 bp long.

Metadata, photos, sequences and trace-files are available in the Barcode of Life Data Systems (BOLD, www.boldsystems.org) through the dataset DS-TRPEX with doi: dx.doi.org/10.5883/DS-TRPEX and dataset DS-CRPEX with doi: dx.doi.org/10.5883/DS-CRPEX. Specimen data and GenBank accession numbers are given in Appendix H.

The barcode sequence of each high-quality sequence (quality score of 80% or above in Sequencher version 4.8) was compared to every COI barcode record with a minimum sequence length of 500 bp in the BOLD Identification System (BOLD-IDS) (Ratnasingham and Hebert 2007) and GenBank's BLAST (Altschul et al. 1990). I accessed the databases on April 7, 2017.

The Fisher's exact test of independence was used to determine whether there were differences in PCR success, sequencing success, and identification success between geographic regions (Norway vs. Costa Rica) or taxonomic groups (subfamily vs. subfamily and tribe vs. tribe) in the R software version 3.3.1 (R Development Core Team 2016). Only taxonomic groups with 10 or more specimens are included in significance tests, as groups with fewer representatives are considered less reliable. Success rates at these three stages (PCR, sequencing, and species identification) was compared to determine when and how failures occurred in the DNA barcoding pipeline. PCR success

was indicated by the presence of a band for each sample on an agarose gel, sequence success by the presence of a high-quality sequence for each sample, and identification success by matching unknown sequences to known chironomid reference sequences with at least 95% similarity in the databases.

Results

Morphological identification

From the Norway pupal exuviae samples, I identified 22 species from 15 genera and eight tribes. Out of the 190 specimens, I identified 109 specimens (57.4%) to described species. However, I identified all 190 specimens (100%) to morphospecies, meaning that differences usually found to be diagnostic on the species-level were observed without finding a match with described species. From the Costa Rica pupal exuviae samples, I identified 22 species from 12 genera and five tribes. Out of the 190 specimens, I identified 14 specimens (7.4%) to species and identified the rest of the specimens to genus (173 specimens, 91.1%) and tribe (3 specimens, 1.6%). However, I identified all 190 specimens (100%) to morphospecies (Table 5.2).

Identification with DNA barcodes

Among the 190 specimens from Norway, 18 specimens (9.5%) failed PCR for COI and could not be sequenced. Of the remaining 172 specimens (90.5%) that had successful PCR, 145 specimens (76.3%) could not be identified as chironomids due to low-quality DNA sequences or non-target contamination. A total of 27 specimens

(14.2%) resulted in high-quality chironomid DNA sequences, which clustered in 10 barcode index numbers (BINs) that closely approximate species of which seven had Linnean names and three interim names. Among the 190 specimens from Costa Rica, 42 specimens (22.1%) failed PCR for COI and could not be sequenced. Of the remaining 148 specimens (77.9%) that had successful PCR, 79 specimens (41.6%) could not be identified as chironomids due to low-quality DNA sequences or non-target contamination. A total of 69 specimens (36.3%) resulted in high-quality chironomid DNA sequences, which clustered in 10 BINs that closely approximate species, but none of these sequences matched known species in BOLD (Table 5.2).

Factors influencing barcoding success: Geographic location

Norwegian pupal exuviae had significantly higher PCR success than the pupal exuviae from Costa Rica ($p = 0.001$), but pupal exuviae from Costa Rica had significantly higher sequence success than those from Norway ($p<0.001$). However, Norwegian pupal exuviae had significantly higher identification success than the pupal exuviae from Costa Rica ($p<0.001$) (Fig. 5.1).

Factors influencing barcoding success: Taxonomic group

There was a significant difference in PCR success ($p = 0.019$), sequence success ($p = 0.013$), and identification success ($p<0.001$) between subfamilies (Table 5.3). Chironominae pupal exuviae had significantly higher PCR success than Prodiamesinae ($p = 0.009$) and Tanypodinae ($p = 0.027$). Orthocladiinae pupal exuviae had significantly

higher PCR success than Prodiamesinae ($p = 0.029$). Chironominae pupal exuviae had significantly higher sequence success than Prodiamesinae ($p = 0.003$). Orthocladiinae had significantly higher sequence success than Chironominae ($p = 0.016$). Chironominae pupal exuviae had significantly higher identification success than Orthocladiinae ($p<0.001$), Prodiamesinae ($p<0.001$), and Tanypodinae ($p<0.001$) (Table 5.3).

There was a significant difference in PCR success ($p = 0.008$), sequence success ($p = 0.038$), and identification success between tribes ($p<0.001$). Tanytarsini had significantly higher PCR success than Corynoneurini ($p = 0.014$), Orthocladiini was significantly higher than Pentaneurini ($p = 0.034$) and Prodiamesinae ($p = 0.024$), and Tanytarsini was significantly higher than Pentaneurini ($p = 0.003$) and Prodiamesinae ($p = 0.002$). Chironomini had significantly higher sequence success than Prodiamesinae ($p = 0.007$) and Tanytarsini had significantly higher sequencing success than Prodiamesinae ($p = 0.006$). Orthocladiini had significantly higher identification success than Chironomini ($p<0.001$), Procladiini ($p<0.001$), Prodiamesinae ($p<0.001$), and Tanytarsini ($p<0.001$). Tanytarsini had significantly higher identification success than Corynoneurini ($p = 0.013$), Pentaneurini ($p = 0.038$), and Prodiamesinae ($p = 0.013$) (Table 5.4 & Fig. 5.2).

Discussion

Currently, species identification using morphology is more than twice as effective than standard DNA barcoding for chironomid pupal exuviae samples. For pupal exuviae samples from both Norway and Costa Rica, I identified 22 species using morphology, but

12 species were missing from the DNA barcoded samples. Similar to my results, Bista et al. (2017) found 10 genera that were identified morphologically, but were neither found in metabarcoding of eDNA or community DNA from samples with chironomid pupal exuviae. I speculate for both studies that morphological identifications are more effective than DNA barcoding because of either missing reference species/genera in the reference library or low DNA quantities for certain taxa. In this study, the presence of non-target species contaminant DNA in the chironomid pupal exuviae samples likely resulted in failed sequencing. Specifically, for the Norway samples, I believe that low barcoding success is related to cross-contamination that was most likely introduced in the field. For example, I found moderate to high densities of a species of water flea (*Holopedium gibberum*) floating on the surface of the water during fieldwork. Cells or free DNA from non-target organisms, like this water flea, could get stuck inside or attached to different parts of the pupal exuviae, contaminate the sample by competing with the low pupal exuviae DNA (Kranzfelder et al. 2016), and reduce the chances that the unknown sequences successfully match known chironomid sequences.

In contrast, I did not observe high densities of non-target species floating on the surface of the water during fieldwork in the Costa Rican sample area. I speculate that the low barcode success for the Costa Rica samples was related to biotic and abiotic environmental factors. Chironomidae pupal exuviae decomposition rates depend on microbial activity, nutrient concentrations, temperature, and turbulence. Specifically, higher microbial (i.e. bacteria and fungi) numbers, warmer waters, higher nutrients levels from polluted waters (i.e. nitrate and ammonia from untreated wastewaters), and higher

turbulence lead to an increase in pupal exuviae decomposition rates (Kavanaugh et al. 2014). In addition, abiotic factors in aquatic habitats, such as ultraviolet radiation, temperature, oxygen, pH, salinity, and substrates, can differ and impact DNA degradation (Barnes et al. 2014; Eichmiller et al. 2016; Strickler et al. 2015). For example, Strickler et al. (2015) found that aquatic habitats that are colder, more protected from solar radiation, and more alkaline have lower DNA degradation rates than those that are warmer, sunnier, and neutral or acidic. Based on these interacting abiotic and biotic environmental factors, I would expect the DNA in Costa Rican pupal exuviae to degrade faster than Norway pupal exuviae.

While the BINs or approximate number of species were the same for both Norway and Costa Rica, the sequencing and identification success rates were different when comparing the two geographic regions. In Norway, the pupal exuviae samples were collected near the Norwegian University of Science and Technology (NTNU) in Trondheim where chironomid barcode projects have been run for almost a decade as part of the Norwegian Barcode of Life (NorBOL). Therefore, it makes sense that all of the high-quality sequences matched known barcoded species. For Costa Rica, I identified all pupal exuviae to morphospecies; however, DNA barcoding did not improve species identifications. I was not able to identify any unknown barcode to known barcoded species, since the reference library, BOLD, lacked public barcode sequences with species identifications from Costa Rica. As of April 7, 2017, BOLD systems had 3,561 chironomid records for Norway representing 112 genera, 552 named species (Linnean and interim names), and 741 BINs. There are currently 631 chironomid species recorded

from Norway (Artsdatabanken 2015) and an estimated 50 undescribed species. Costa Rica had 17,482 chironomid records in BOLD. There are currently 51 species recorded (Spies and Reiss 1996), but there are an estimated 1,000 undescribed species within the Dr. William P. Coffman Costa Rica collection at La Selva Biological Station (de la Rosa 2015) and perhaps 2,000 species in the country (C.L. de la Rosa, personal communication). My results indicate that association of the Costa Rican specimens to named species suffers from the incompleteness of the barcode reference library for Chironomidae from this region. While many Costa Rican chironomids have been barcoded, most of these barcodes are not identified to species due to the lack of expertise in Costa Rican chironomid taxonomy. Reliable species identification of chironomids with DNA barcoding requires the presence of named species in the reference library (Ekrem et al. 2007) and current reference libraries are still largely incomplete in terms of both species and geographic ranges.

In addition to differences in barcoding success related to geographic regions, I found significant differences in DNA barcoding success when comparing taxonomic groups (subfamilies and tribes). Subfamily Chironominae, and more specifically tribe Tanytarsini, had the highest PCR and identification success for both Norway and Costa Rica samples. These results are not surprising since I have observed Tanytarsini specimens struggle to emerge as adults from their pupal skins during individual rearing, possibly leaving behind higher numbers of epithelial cells and other cell-based structures from the adult (the cuticle itself is extracellular). Also, decomposition rates of pupal exuviae are impacted by the degree of chitinization of the species with lightly chitinized

exuviae sinking faster (Kavanaugh et al. 2014). Tanytarsini tend to be darker and more sclerotized than other tribes, which would reduce their decomposition rate and increase the chances of trace DNA being available for DNA extraction. As a result, researchers that would like to identify chironomid pupal exuviae to species must consider the influence of environmental conditions in different geographic regions and the physical structures of various taxonomic groups on the preservation of DNA when designing their DNA barcoding studies.

DNA barcoding allows the inclusion of all life stages in biodiversity assessments (Ekrem et al. 2010) and collections of chironomid pupal exuviae are commonly used for biological monitoring of water quality (Kranzfelder et al. 2015). However, DNA barcoding of chironomid pupal exuviae alone is not currently effective for species identification of unknown specimens. Chironomid pupal exuviae samples have low quantities of DNA and are easily contaminated by non-target species DNA or degraded by biotic or abiotic environmental factors, like temperature, UV-light, or acidity. Also, public reference libraries, like BOLD, are not yet sufficiently populated by reference sequences with species names, especially from Costa Rica. Therefore, I recommend future biodiversity studies that focus their efforts on understudied Neotropical regions, like Costa Rica, to simultaneously use both morphological and molecular identification techniques to identify all life stages of chironomids. This way, gaps in the reference library can be filled, life stages associated, and morphological characteristics evaluated simultaneously.

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Tables and Figures

Table 5.1: Locality information for chironomid pupal exuviae collections. All Norway and Costa Rica samples were collected in 2014.

a. Norway			
Sample site	Aquatic system	GPS	Sample date(s)
Lianvatnet	Lake	N 63.403°, E 10.318°	Aug. 31
Nidelva	River	N 63.429°, E 10.379°	Sept. 5, 12, 17
b. Costa Rica			
Sample site	Aquatic system	GPS	Sample date(s)
Rio Grande	River	N 9.635°, W 82.678	Dec. 13
Quebrada Dos Aguas	Stream below a waterfall	N 9.631°, W 82.819°	Dec. 14
Río Negro	River	N 9.644°, W 82.732°	Dec. 15
Río Cocles	River	N 9.646°, W 82.735°	Dec. 15
Rio Punta Uva	River	N 9.636°, W 82.694°	Dec. 15
Rio Manzanillo	River	N 9.628°, W 82.677°	Dec. 15

Table 5.2: List of species and number of pupal exuviae identified using morphology and DNA barcoding from Norway and Costa Rica.

a. Norway		
Species	Morphology	DNA barcoding
Procladius (*Holotanypus*) *signatus* (Zetterstedt, 1850)	2	1
Rheopelopia maculipennis (Zetterstedt, 1838)	8	
Potthastia gaedii (Meigen, 1838)	2	
Prodiamesa olivacea (Meigen, 1818)	20	1
Corynoneura sp. 1PK	1	
Corynoneura sp. 15ES	1	1
Cricotopus sp. 1PK	12	
Cricotopus cf. *septentrionalis* Hirvenjoa, 1973	4	4
Cricotopus cf. *similis* Goetghebuer, 1921	20	
Eukiefferiella sp. 1PK	40	
Orthocladius sp. 1PK	2	1
Orthocladius (*Orthocladius*) *oblidens* (Walker, 1856)	18	1
Thienemanniella sp. 1PK	8	
Tvetenia calvescens (Edwards, 1929)	20	4
Chironomus sp. 1PK	2	2
Chironomus (*Chironomus*) cf. *tenuistylus* Brundin, 1949	5	3
Demicryptochironomus sp. 1PK	1	
Micropsectra sp. 1PK	11	
Micropsectra sp. 5ES	1	1
Micropsectra logani (Johannsen, 1928)	8	8
Polypedilum sp. 1PK	2	
Tanytarsus aculeatus Brundin, 1949	2	
Total	**190**	**27**

b. Costa Rica		
Species	Morphology	DNA barcoding
Zavrelimyia sp. 1PK	1	1
Corynoneura sp. 1PK	1	
Onconeura cf. *semifimbriata* Sæther, 1981	5	
Onconeura cf. *similispina* Wiedenbrug, Mendes, Pepinelli & Trivinho-Strixino, 2009	2	2
Onconeura cf. *japi* Wiedenbrug, Mendes, Pepinelli & Trivinho-Strixino, 2009	2	
Thienemanniella sp. 1PK	1	
Cricotopus (*Isocladius*) sp. 1PK	139	57
Cricotopus (*Cricotopus*) sp. 2PK	4	
Cricotopus (*Cricotopus*) sp. 3PK	2	

b. Costa Rica		
Species	Morphology	DNA barcoding
Cricotopus (*Cricotopus*) sp. 4PK	1	
Cricotopus (*Cricotopus*) sp. 5PK	1	
Cricotopus (*Isocladius*) sp. 6PK	2	
Cricotopus (*Nostococladius*) sp. 7PK	2	
Nanocladius (*Nanocladius*) sp. 1PK	2	2
Nanocladius (*Nanocladius*) sp. 2PK	2	
Parametriocnemus sp. 1PK	14	2
Apedilum cf. *elachistus* Townes, 1945	5	2
Polypedilum sp. 1PK	1	
Polypedilum sp. 2PK	1	
Chironomini Genus A Jacobsen, 2008	1	1
Tanytarsini #1PK	1	
Tanytarsini #2PK	1	1
Total	**190**	**69**

Table 5.3: Comparison of PCR, sequence, and identification success by subfamily for pooled samples from Norway and Costa Rica.

Subfamily	No. of pupal exuviae	PCR success (%)	Sequence success (%)	Identification success (%)
Tanypodinae	11	7 (64)	2 (18)	1 (9)
Prodiamesinae	20	13 (65)	1 (5)	1 (5)
Orthocladiinae	305	259 (85)	75 (25)	11 (4)
Chironominae	42	39 (93)	18 (43)	14 (33)

Table 5.4: Comparison of PCR, sequence, and identification success by tribe for pooled samples from Norway and Costa Rica. Prodiamesinae is identified at the subfamily level, since there are no tribes within this subfamily.

Subfamily	No. of pupal exuviae	PCR success (%)	Sequence success (%)	Identification success (%)
Prodiamesinae	20	13 (65)	1 (5)	1 (5)
Corynoneurini	20	15 (75)	4 (20)	1 (5)
Orthocladiini	285	244 (86)	71 (25)	10 (4)
Tanytarsini	24	24 (100)	10 (42)	9 (38)
Chironomini	18	15 (83)	8 (44)	5 (28)

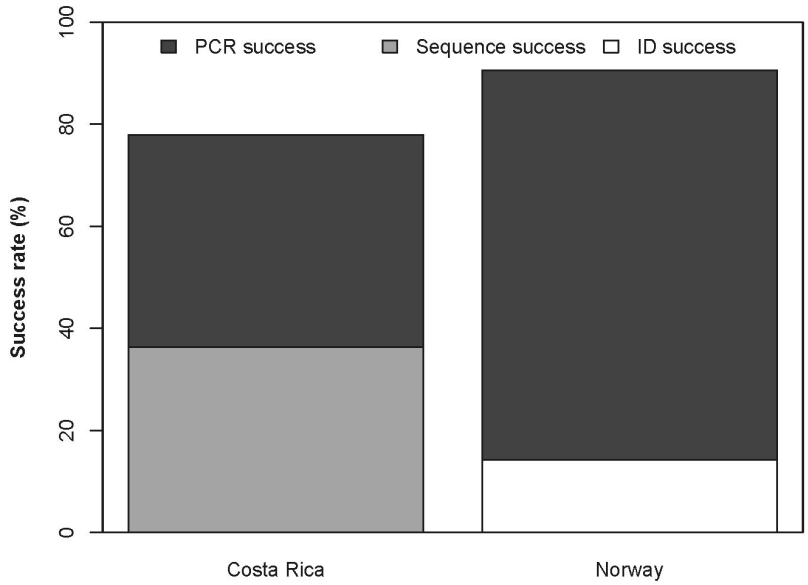

Figure 5.1: Percent PCR, sequence, and identification (ID) success rate of Costa Rican versus Norwegian pupal exuviae. Norway sequence success was equal to identification success.

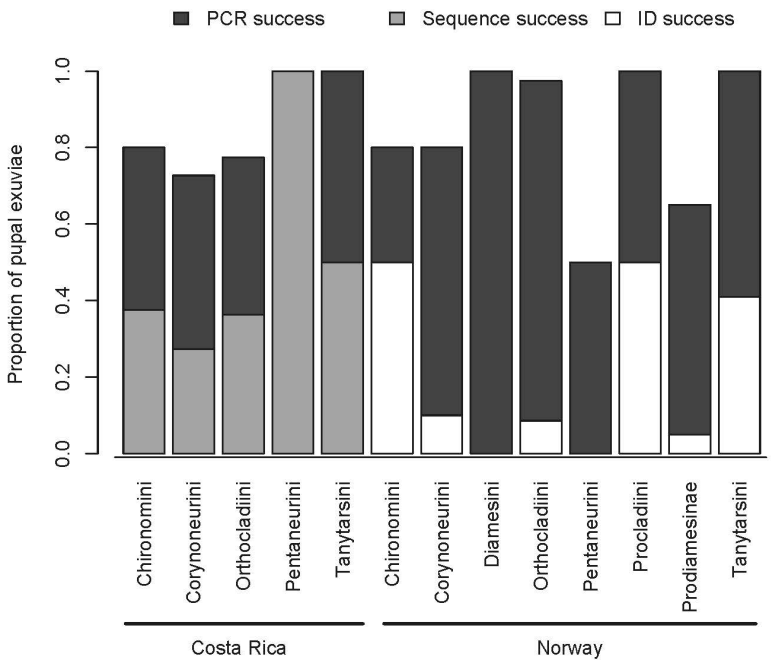

Figure 5.2: Comparison of the proportion of pupal exuviae with successful PCR, sequence, and identification by tribe with DNA barcoding for Costa Rica and Norway. Prodiamesinae is identified at the subfamily level, since there are no tribes within this subfamily.

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APPENDICES

Appendix A: Summary of Landsat classification accuracies (%) for six watersheds in 2001. *PA* Producer's Accuracy, *UA* User's Accuracy, *WA* Water class, *FO* Forest class, *RC* Row crop class, *PA* Pasture class, *UR* Urban class. Kappa is expressed as a decimal.

	Chirripo		Tortuguero		Reventazón		Pacuare		Bananito		Estrella	
	PA	UA	PA	UA	PA	UA	PA	UA	PA	UA	PA	UA
WA	92	100	93	100	96	99	80	98	64	100	76	98
FO	96	74	97	86	93	89	100	74	97	65	100	66
RC	77	91	99	86	81	69	93	81	95	79	95	96
PA	95	83	87	86	79	72	77	76	60	74	72	76
UR	80	100	76	98	68	98	67	100	56	67	67	85
Overall	88		90		84		84		74		82	
Kappa	0.85		0.88		0.79		0.79		0.68		0.77	

Appendix B: Summary of Landsat classification accuracies (%) for six watersheds in 2014. *PA* Producer's Accuracy, *UA* User's Accuracy, *WA* Water class, *FO* Forest class, *RC* Row crop class, *PA* Pasture class, *UR* Urban class. Kappa is expressed as a decimal.

	Chirripo		Tortuguero		Reventazón		Pacuare		Bananito		Estrella	
	PA	UA	PA	UA	PA	UA	PA	UA	PA	UA	PA	UA
WA	91	100	83	98	76	100	96	99	55	100	47	100
FO	99	62	96	67	97	81	99	80	100	54	95	52
RC	64	87	73	80	77	67	93	76	89	86	80	81
PA	81	78	83	81	80	74	71	79	69	78	69	72
UR	67	91	75	95	76	95	67	98	57	88	61	81
Overall	80		82		81		85		74		70	
Kappa	0.75		0.77		0.77		0.81		0.67		0.63	

Appendix C: Table of Specific Materials/Equipment.

Name of Material/Equipment	Company	Catalog Number	Comments/Description
Ethanol	Fisher Scientific	S25309B	70-95%
Plastic wash bottles	Fisher Scientific	0340923B	
Sample jar	Fisher Scientific	0333510B	Glass or plastic, 60-mL recommended
Testing sieve	Advantech	120SS12F	125-micron mesh size
Larval tray	BioQuip	5524	White
Stereo microscope			
Glass shell vials	Fisher Scientific	0333926B	1-dram size
Plastic dropper	Thermo Scientific	1371110	30 to 35 drops/mL
Fine forceps	BioQuip	4524	#5
Petri dish	Carolina	741158	Glass or plastic
Multi-well plate	Thermo Scientific	144530	Glass or plastic
Glass microslides	Thermo Scientific	3010002	3 x 1 in.
Glass cover slips	Thermo Scientific	12-519-21G	Circular or square
Euparal mounting medium	BioQuip	6372B	
Pigma pen	BioQuip	1154F	Black
Probe	BioQuip	4751	
Kimwipes	Kimberly-Clark Professional™	34120	

Appendix D: List of Chironomidae species collected from nine estuaries on the Caribbean coast of Costa Rica from 2012-2014.

Subfamily	Tribe	Genus	Subgenus	Species	Author
Tanypodinae	Coelotanypodini	*Coelotanypus*		sp. 1	
Tanypodinae	Coelotanypodini	*Coelotanypus*		sp. 2	
Tanypodinae	Coelotanypodini	*Coelotanypus*		sp. 3	
Tanypodinae	Macropelopiini	*Fittkauimyia*		sp. 1	
Tanypodinae	Pentaneurini	*Ablabesmyia*		sp. 1	
Tanypodinae	Pentaneurini	*Ablabesmyia*		sp. 2	
Tanypodinae	Pentaneurini	*Ablabesmyia*		sp. 3	
Tanypodinae	Pentaneurini	*Ablabesmyia*		sp. 4	
Tanypodinae	Pentaneurini	*Ablabesmyia*		sp. 5	
Tanypodinae	Pentaneurini	*Ablabesmyia*		sp. 6	
Tanypodinae	Pentaneurini	*Labrundinia*		sp. 1	
Tanypodinae	Pentaneurini	*Labrundinia*		sp. 2	
Tanypodinae	Pentaneurini	*Labrundinia*		sp. 3	
Tanypodinae	Pentaneurini	*Labrundinia*		sp. 4	
Tanypodinae	Pentaneurini	*Labrundinia*		sp. 5	
Tanypodinae	Pentaneurini	*Labrundinia*		sp. 6	
Tanypodinae	Pentaneurini	*Larsia*		sp. 1	
Tanypodinae	Pentaneurini	*Larsia*		sp. 2	
Tanypodinae	Pentaneurini	*Monopelopia*		sp. 1	
Tanypodinae	Pentaneurini	*Monopelopia*		sp. 2	
Tanypodinae	Pentaneurini	*Nilotanypus*		sp. 1	
Tanypodinae	Pentaneurini	*Nilotanypus*		cf. *kansensis*	Roback, 1986
Tanypodinae	Pentaneurini	*Pentaneura*		sp. 1	
Tanypodinae	Pentaneurini	*Zavrelimyia*		sp. 1	
Tanypodinae	Procladiini	*Procladius*	Psilotanypus	sp. 1	
Tanypodinae	Tanypodini	*Tanypus*	Apelopia	sp. 1	
Orthocladiinae	Corynoneurini	*Corynoneura*		sp. 1	
Orthocladiinae	Corynoneurini	*Corynoneura*		sp. 2	
Orthocladiinae	Corynoneurini	*Corynoneura*		sp. 3	
Orthocladiinae	Corynoneurini	*Corynoneura*		cf. *sisbiota*	Wiedenbrug, Lamas & Trivinho-Strixino, 2012
Orthocladiinae	Corynoneurini	*Corynoneura*		sp. 4	
Orthocladiinae	Corynoneurini	*Corynoneura*		sp. 5	
Orthocladiinae	Corynoneurini	*Corynoneura*		sp. 6	
Orthocladiinae	Corynoneurini	*Onconeura*		sp. 1	
Orthocladiinae	Corynoneurini	*Onconeura*		sp. 2	
Orthocladiinae	Corynoneurini	*Onconeura*		sp. 3	
Orthocladiinae	Corynoneurini	*Thienemanniella*		sp. 1	
Orthocladiinae	Corynoneurini	*Thienemanniella*		sp. 2	
Orthocladiinae	Corynoneurini	*Thienemanniella*		sp. 3	
Orthocladiinae	Corynoneurini	*Thienemanniella*		sp. 4	
Orthocladiinae	Corynoneurini	*Thienemanniella*		sp. 5	
Orthocladiinae	Corynoneurini	*Thienemanniella*		sp. 6	
Orthocladiinae	Corynoneurini	*Thienemanniella*		sp. 7	
Orthocladiinae	Corynoneurini	*Thienemanniella*		sp. 8	
Orthocladiinae	Corynoneurini	*Ubatubaneura*		sp. 1	
Orthocladiinae	Corynoneurini	*Ubatubaneura*		sp. 2	
Orthocladiinae	Corynoneurini	*Ubatubaneura*		sp. 3	
Orthocladiinae	Corynoneurini	*Ubatubaneura*		sp. 4	

Subfamily	Tribe	Genus	Subgenus	Species	Author
Orthocladiinae	Orthocladiini	*Cricotopus*	*Cricotopus*	sp. 1	
Orthocladiinae	Orthocladiini	*Cricotopus*	*Cricotopus*	sp. 2	
Orthocladiinae	Orthocladiini	*Cricotopus*	*Cricotopus*	sp. 3	
Orthocladiinae	Orthocladiini	*Cricotopus*	*Cricotopus*	sp. 4	
Orthocladiinae	Orthocladiini	*Cricotopus*	*Cricotopus*	sp. 5	
Orthocladiinae	Orthocladiini	*Cricotopus*	*Cricotopus*	sp. 6	
Orthocladiinae	Orthocladiini	*Cricotopus*	*Cricotopus*	sp. 7	
Orthocladiinae	Orthocladiini	*Cricotopus*	*Cricotopus*	sp. 8	
Orthocladiinae	Orthocladiini	*Cricotopus*	*Cricotopus*	sp. 9	
Orthocladiinae	Orthocladiini	*Cricotopus*	*Cricotopus*	sp. 10	
Orthocladiinae	Orthocladiini	*Cricotopus*	*Nostococladius*	sp. 1	
Orthocladiinae	Orthocladiini	*Cricotopus*	*Nostococladius*	sp. 2	
Orthocladiinae	Orthocladiini	*Cricotopus*	*Oliveiriella*	sp. 1	
Orthocladiinae	Orthocladiini	*Eukiefferiella*		sp. 1	
Orthocladiinae	Orthocladiini	*Eukiefferiella*		sp. 2	
Orthocladiinae	Orthocladiini	*Eukiefferiella*		sp. 3	
Orthocladiinae	Orthocladiini	*Eukiefferiella*		sp. 4	
Orthocladiinae	Orthocladiini	*Gymnometriocnemus*		sp. 1	
Orthocladiinae	Orthocladiini	*Gymnometriocnemus*		sp. 2	
Chironominae	Chironomini	*Microchironomus*		sp. 1	
Orthocladiinae	Orthocladiini	*Nanocladius*	*Nanocladius*	sp. 1	
Orthocladiinae	Orthocladiini	*Nanocladius*	*Nanocladius*	sp. 2	
Orthocladiinae	Orthocladiini	*Nanocladius*	*Nanocladius*	sp. 3	
Orthocladiinae	Orthocladiini	*Orthocladius*	*Orthocladius*	sp. 1	
Orthocladiinae	Orthocladiini	*Orthocladius*	*Orthocladius*	sp. 2	
Orthocladiinae	Orthocladiini	*Orthocladius*	*Orthocladius*	sp. 3	
Orthocladiinae	Orthocladiini	*Parakiefferiella*		sp. 1	
Orthocladiinae	Orthocladiini	*Parakiefferiella*		sp. 2	
Orthocladiinae	Orthocladiini	*Parametriocnemus*		sp. 1	
Orthocladiinae	Orthocladiini	*Parametriocnemus*		sp. 2	
Orthocladiinae	Orthocladiini	*Pseudosmittia*		*trilobata*	(Edwards, 1929)
Orthocladiinae	Orthocladiini	*Stictocladius*		sp. 1	
Orthocladiinae				sp. 1	
Orthocladiinae				sp. 2	
Orthocladiinae				sp. 3	
Chironominae	Chironomini	*Apedilum*		sp. 1	
Chironominae	Chironomini	*Axarus*		sp. 1	
Chironominae	Chironomini	*Axarus*		sp. 2	
Chironominae	Chironomini	*Beardius*		cf. *reissi*	Jacobsen & Perry, 2008
Chironominae	Chironomini	*Chironomus*		sp. 1	
Chironominae	Chironomini	*Chironomus*		sp. 2	
Chironominae	Chironomini	*Chironomus*		sp. 3	
Chironominae	Chironomini	*Cladopelma*		cf. *forcipis*	(Rempel, 1939)
Chironominae	Chironomini	*Cladopelma*		sp. 1	
Chironominae	Chironomini	*Cryptochironomus*		sp. 1	
Chironominae	Chironomini	*Cryptochironomus*		sp. 2	
Chironominae	Chironomini	*Cryptochironomus*		sp. 3	
Chironominae	Chironomini	*Cryptochironomus*		sp. 4	
Chironominae	Chironomini	*Cryptochironomus*		sp. 5	
Chironominae	Chironomini	*Cryptochironomus*		sp. 6	
Chironominae	Chironomini	*Cryptochironomus*		sp. 7	
Chironominae	Chironomini	*Cryptochironomus*		sp. 8	
Chironominae	Chironomini	*Cryptochironomus*		sp. 9	
Chironominae	Chironomini	*Cryptochironomus*		sp. 10	

Subfamily	Tribe	Genus	Subgenus	Species	Author
Chironominae	Chironomini	*Cryptotendipes*		sp. 1	
Chironominae	Chironomini	*Cryptotendipes*		sp. 2	
Chironominae	Chironomini	*Dicrotendipes*		sp. 1	
Chironominae	Chironomini	*Dicrotendipes*		sp. 2	
Chironominae	Chironomini	*Dicrotendipes*		sp. 3	
Chironominae	Chironomini	*Dicrotenidpes*		sp. 4	
Chironominae	Chironomini	*Einfeldia*		sp. 1	
Chironominae	Chironomini	*Endotribelos*		sp. 1	
Chironominae	Chironomini	*Endotribelos*		sp. 2	
Chironominae	Chironomini	*Endotribelos*		sp. 3	
Chironominae	Chironomini	*Endotribelos*		sp. 4	
Chironominae	Chironomini	*Endotribelos*		sp. 5	
Chironominae	Chironomini	*Endotribelos*		sp. 6	
Chironominae	Chironomini	*Goeldichironomus*		sp. 1	
Chironominae	Chironomini	*Goeldichironomus*		sp. 2	
Chironominae	Chironomini	*Harnischia* complex		sp. 1	
Chironominae	Chironomini	*Harnischia* complex		sp. 2	
Chironominae	Chironomini	*Harnischia* complex		sp. 3	
Chironominae	Chironomini	*Harnischia* complex		sp. 4	
Chironominae	Chironomini	*Harnischia* complex		sp. 5	
Chironominae	Chironomini	*Nilothauma*		cf. *reissi*	(Soponis, 1987)
Chironominae	Chironomini	*Nilothauma*		sp. 1	
Chironominae	Chironomini	*Nilothauma*		sp. 2	
Chironominae	Chironomini	*Nilothauma*		sp. 3	
Chironominae	Chironomini	*Nilothauma*		sp. 4	
Chironominae	Chironomini	*Parachironomus*		sp. 1	
Chironominae	Chironomini	*Parachironomus*		sp. 2	
Chironominae	Chironomini	*Parachironomus*		sp. 3	
Chironominae	Chironomini	*Parachironomus*		sp. 4	
Chironominae	Chironomini	*Parachironomus*		sp. 5	
Chironominae	Chironomini	*Parachironomus*		sp. 6	
Chironominae	Chironomini	*Parachironomus*		sp. 7	
Chironominae	Chironomini	*Parachironomus*		sp. 8	
Chironominae	Chironomini	*Parachironomus*		sp. 9	
Chironominae	Chironomini	*Paralauterborniella*		sp. 1	
Chironominae	Chironomini	*Paralauterborniella*		sp. 2	
Chironominae	Chironomini	*Polypedilum*	*Asheum*	sp. 1	
Chironominae	Chironomini	*Polypedilum*		sp. 2	
Chironominae	Chironomini	*Polypedilum*		sp. 3	
Chironominae	Chironomini	*Polypedilum*		sp. 4	
Chironominae	Chironomini	*Polypedilum*	*Tripodura*	sp. 5	
Chironominae	Chironomini	*Polypedilum*		sp. 6	
Chironominae	Chironomini	*Polypedilum*		sp. 7	
Chironominae	Chironomini	*Polypedilum*		sp. 8	
Chironominae	Chironomini	*Polypedilum*	*Tripodura*	sp. 9	
Chironominae	Chironomini	*Polypedilum*		sp. 10	
Chironominae	Chironomini	*Polypedilum*	*Asheum*	sp. 11	
Chironominae	Chironomini	*Polypedilum*		sp. 12	
Chironominae	Chironomini	*Polypedilum*		sp. 13	
Chironominae	Chironomini	*Polypedilum*		sp. 14	
Chironominae	Chironomini	*Polypedilum*		sp. 15	
Chironominae	Chironomini	*Polypedilum*		sp. 16	
Chironominae	Chironomini	*Polypedilum*		sp. 17	
Chironominae	Chironomini	*Polypedilum*		sp. 18	
Chironominae	Chironomini	*Polypedilum*		sp. 19	

Subfamily	Tribe	Genus	Subgenus	Species	Author
Chironominae	Pseudochironomini	*Pseudochironomus*		sp. 1	
Chironominae	Pseudochironomini	*Pseudochironomus*		sp. 2	
Chironominae	Pseudochironomini	*Pseudochironomus*		sp. 3	
Chironominae	Pseudochironomini	*Pseudochironomus*		sp. 4	
Chironominae	Chironomini	*Robackia*		cf. *claviger*	(Townes, 1945)
Chironominae	Chironomini	*Robackia*		sp. 1	
Chironominae	Chironomini	*Saetheria*		sp. 1	
Chironominae	Chironomini	*Saetheria*		sp. 2	
Chironominae	Chironomini	*Saetheria*		sp. 3	
Chironominae	Chironomini	*Stenochironomus*		sp. 1	
Chironominae	Chironomini	*Stenochironomus*		sp. 2	
Chironominae	Chironomini	*Stenochironomus*		sp. 3	
Chironominae	Chironomini	*Stenochironomus*		sp. 4	
Chironominae	Chironomini	*Stenochironomus*		sp. 5	
Chironominae	Chironomini	*Stenochironomus*		sp. 6	
Chironominae	Chironomini	*Stenochironomus*		sp. 7	
Chironominae	Chironomini	*Stenochironomus*		sp. 8	
Chironominae	Chironomini	*Stenochironomus*		sp. 9	
Chironominae	Chironomini	*Stictochironomus*		sp. 1	
Chironominae	Chironomini	*Xenochironomus*		cf. *xenolabis*	(Kieffer, 1916)
Chironominae	Chironomini	*Xestochironomus*		sp. 1	
Chironominae	Chironomini	*Xestochironomus*		sp. 2	
Chironominae	Chironomini	*Xestochironomus*		sp. 3	
Chironominae	Chironomini	*Xestochironomus*		sp. 4	
Chironominae	Chironomini	*Zavreliella*		sp. 1	
Chironominae	Chironomini	*Zavreliella*		sp. 2	
Chironominae	Chironomini			sp. 2	
Chironominae	Chironomini			sp. 3	
Chironominae	Chironomini			sp. 4	
Chironominae	Chironomini			sp. 5	
Chironominae	Chironomini			sp. 7	
Chironominae	Chironomini			sp. 8	
Chironominae	Chironomini			sp. 9	
Chironominae	Chironomini			sp. 10	
Chironominae	Chironomini			sp. 11	
Chironominae	Chironomini			sp. 12	
Chironominae	Chironomini			sp. 13	
Chironominae	Tanytarsini	*Cladotanytarsus*		sp. 1	
Chironominae	Tanytarsini	*Cladotanytarsus*		sp. 2	
Chironominae	Tanytarsini	*Cladotanytarsus*		sp. 3	
Chironominae	Tanytarsini	*Paratanytarsus*		sp. 1	
Chironominae	Tanytarsini	*Rheotanytarsus*		sp. 1	
Chironominae	Tanytarsini	*Rheotanytarsus*		sp. 2	
Chironominae	Tanytarsini	*Stempellina*		sp. 1	
Chironominae	Tanytarsini	*Stempellinella*		sp. 1	
Chironominae	Tanytarsini	*Stempellinella*		sp. 2	
Chironominae	Tanytarsini	*Sublettea*		sp. 1	
Chironominae	Tanytarsini	*Tanytarsus*		sp. 1	
Chironominae	Tanytarsini	*Tanytarsus*		sp. 2	
Chironominae	Tanytarsini	*Tanytarsus*		sp. 3	
Chironominae	Tanytarsini	*Tanytarsus*		sp. 4	
Chironominae	Tanytarsini	*Tanytarsus*		sp. 5	
Chironominae	Tanytarsini	*Tanytarsus*		sp. 6	
Chironominae	Tanytarsini	*Tanytarsus*		sp. 7	

Subfamily	Tribe	Genus	Subgenus	Species	Author
Chironominae	Tanytarsini	*Tanytarsus*		sp. 8	
Chironominae	Tanytarsini	*Tanytarsus*		sp. 9	
Chironominae	Tanytarsini	*Tanytarsus*		sp. 10	
Chironominae	Tanytarsini	*Tanytarsus*		sp. 11	
Chironominae	Tanytarsini	*Tanytarsus*		sp. 12	
Chironominae	Tanytarsini	*Tanytarsus*		sp. 13	
Chironominae	Tanytarsini	*Tanytarsus*		sp. 14	
Chironominae	Tanytarsini	*Tanytarsus*		sp. 15	
Chironominae	Tanytarsini	*Tanytarsus*		sp. 16	
Chironominae	Tanytarsini	*Tanytarsus*		sp. 17	
Chironominae	Tanytarsini	*Tanytarsus*		sp. 18	
Chironominae	Tanytarsini	*Tanytarsus*		sp. 19	
Chironominae	Tanytarsini	*Tanytarsus*		sp. 20	
Chironominae	Tanytarsini	*Tanytarsus*		sp. 21	
Chironominae	Tanytarsini	*Tanytarsus*		sp. 22	
Chironominae	Tanytarsini	*Tanytarsus*		sp. 23	
Chironominae	Tanytarsini	*Tanytarsus*		sp. 24	

Appendix E: Number of individuals present in each estuary.

Species	Laguna Barra del Colorado	Laguna Cuatro	Laguna del Tortuguero	Laguna Jalova	Río Parismina	Río Pacuare	Estero Negro	Río Estrella	Río Bananito	**Total**
Ablabesmyia sp. 1	1	0	0	0	0	0	0	0	0	1
Ablabesmyia sp. 2	0	0	0	0	0	1	0	0	0	1
Ablabesmyia sp. 3	0	0	1	0	0	0	0	3	5	9
Ablabesmyia sp. 4	0	0	2	0	0	0	1	3	1	7
Ablabesmyia sp. 5	0	0	2	0	0	0	1	0	0	3
Ablabesmyia sp. 6	0	0	0	0	0	0	0	0	1	1
Apedilum sp. 1	1	0	0	0	0	7	0	13	16	37
Axarus sp. 1	433	0	0	0	0	3	0	0	0	436
Axarus sp. 2	0	0	0	0	0	0	0	1	5	6
Beardius cf. *reissi*	0	18	7	2	0	0	0	3	0	30
Chironomini sp. 10	0	0	0	1	0	0	0	0	0	1
Chironomini sp. 11	2	6	1	4	0	0	0	0	5	18
Chironomini sp. 12	0	0	0	0	0	0	0	0	1	1
Chironomini sp. 13	0	0	1	11	0	0	0	0	1	13
Chironomini sp. 2	6	0	0	0	15	121	0	0	0	142
Chironomini sp. 3	1	0	0	0	4	37	0	0	0	42
Chironomini sp. 4	4	0	0	0	143	98	0	0	0	245
Chironomini sp. 5	0	0	0	0	0	0	0	12	3	15
Chironomini sp. 7	0	0	0	1	0	0	0	0	3	4
Chironomini sp. 8	0	0	0	0	0	0	0	0	1	1
Chironomini sp. 9	26	0	0	0	0	29	0	8	0	63
Chironomus sp. 1	0	3	4	0	0	0	0	3	0	10
Chironomus sp. 2	0	0	3	1	0	0	0	0	0	4
Chironomus sp. 3	0	66	0	1	0	0	0	0	1	68
Cladopelma cf. *forcipis*	0	0	0	0	0	0	0	0	2	2
Cladopelma sp. 1	0	147	0	0	0	0	0	1	2	150
Cladotanytarsus sp. 1	0	0	0	0	0	6	0	217	179	402
Cladotanytarsus sp. 2	1	0	0	0	0	0	0	2	0	3
Cladotanytarsus sp. 3	0	0	0	576	0	0	0	12	0	588
Coelotanypus sp. 1	3	0	90	4	0	2	0	30	31	160
Coelotanypus sp. 2	0	0	12	0	0	1	0	0	0	13
Coelotanypus sp. 3	0	0	26	59	0	10	0	0	0	95
Corynoneura cf. *sisbiota*	0	0	0	0	0	0	0	0	45	45
Corynoneura sp. 1	0	1	5	0	1	0	1	1	30	39
Corynoneura sp. 2	2	0	1	0	1	12	0	2	28	46

Species	Laguna Barra del Colorado	Laguna Cuatro	Laguna del Tortuguero	Laguna Jalova	Río Parismina	Río Pacuare	Estero Negro	Río Estrella	Río Bananito	Total
Corynoneura sp. 3	0	0	1	0	0	0	0	0	0	1
Corynoneura sp. 4	0	0	0	0	0	0	0	0	1	1
Corynoneura sp. 5	0	0	0	0	1	0	0	0	0	1
Corynoneura sp. 6	0	0	0	0	0	0	0	0	1	1
Cricotopus (*Cricotopus*) sp. 1	95	15	59	90	9	34	2	493	124	921
Cricotopus (*Cricotopus*) sp. 10	0	0	0	0	0	1	0	6	7	14
Cricotopus (Cricotopus) sp. 2	1	0	0	0	0	1	0	17	43	62
Cricotopus (*Cricotopus*) sp. 3	2	0	0	0	0	1	2	93	113	211
Cricotopus (*Cricotopus*) sp. 4	0	0	0	0	0	4	0	1	0	5
Cricotopus (*Cricotopus*) sp. 5	1	0	0	0	0	1	0	57	42	101
Cricotopus (*Cricotopus*) sp. 6	0	0	0	0	0	0	0	1	0	1
Cricotopus (*Cricotopus*) sp. 7	0	0	0	1	0	1	0	54	25	81
Cricotopus (*Cricotopus*) sp. 8	0	0	0	0	0	4	0	4	0	8
Cricotopus (*Cricotopus*) sp. 9	0	0	0	0	0	0	0	14	30	44
Cricotopus (*Nostococladius*) sp. 1	177	0	0	0	0	0	0	0	0	177
Cricotopus (*Nostococladius*) sp. 2	0	0	0	0	0	0	0	1	0	1
Cricotopus (*Oliveiriella*) sp. 1	0	0	0	0	0	0	0	1	0	1
Cryptochironomus sp. 1	0	0	17	39	1	9	0	240	20	326
Cryptochironomus sp. 10	1	0	0	0	0	0	0	0	0	1
Cryptochironomus sp. 2	0	0	0	0	0	0	0	5	2	7
Cryptochironomus sp. 3	0	0	0	1	0	0	0	0	0	1
Cryptochironomus sp. 4	3	0	0	0	0	0	0	0	0	3
Cryptochironomus sp. 5	19	0	0	0	0	2	0	5	3	29
Cryptochironomus sp. 6	0	0	0	0	1	0	0	0	0	1
Cryptochironomus sp. 7	0	1	0	2	0	0	0	0	0	3
Cryptochironomus sp. 8	2	0	0	1	2	1	0	0	0	6
Cryptochironomus sp. 9	0	0	0	0	0	0	0	0	1	1
Cryptotendipes sp. 1	5	0	31	137	12	111	0	79	468	843
Cryptotendipes sp. 2	0	0	0	0	0	0	0	1	0	1
Dicrotendipes sp. 1	0	29	4	1	0	0	14	8	47	103
Dicrotendipes sp. 2	0	0	3	1	0	0	0	118	25	147
Dicrotendipes sp. 3	0	0	0	0	0	0	0	4	0	4
Dicrotendipes sp. 4	0	0	1	0	0	0	0	0	0	1
Einfeldia sp. 1	0	3	0	0	0	0	0	0	0	3
Endotribelos sp. 1	0	0	0	0	0	1	0	3	1	5
Endotribelos sp. 2	1	1	4	11	0	1	2	15	21	56
Endotribelos sp. 3	0	0	0	0	0	0	0	0	1	1

Species	Laguna Barra del Colorado	Laguna Cuatro	Laguna del Tortuguero	Laguna Jalova	Río Parismina	Río Pacuare	Estero Negro	Río Estrella	Río Bananito	Total
Endotribelos sp. 4	0	0	2	0	0	0	0	0	0	2
Endotribelos sp. 5	0	0	0	0	0	0	0	0	2	2
Endotribelos sp. 6	0	1	0	0	0	0	0	0	0	1
Eukiefferiella sp. 1	0	0	0	0	3	5	0	1	0	9
Eukiefferiella sp. 2	0	0	0	0	0	1	0	0	0	1
Eukiefferiella sp. 3	0	0	1	0	0	0	0	0	0	1
Eukiefferiella sp. 4	0	0	0	0	0	0	0	1	0	1
Fittkauimyia sp. 1	0	1	0	0	0	0	0	0	0	1
Goeldichironomus sp. 1	0	0	0	0	0	0	3	0	0	3
Goeldichironomus sp. 2	0	2	0	0	0	0	0	0	0	2
Gymnometriocnemus sp. 1	1	0	0	0	0	1	1	0	1	4
Gymnometriocnemus sp. 2	1	0	0	0	0	1	0	0	0	2
Harnischia complex sp. 1	12	0	0	0	225	201	0	1	0	439
Harnischia complex sp. 2	72	0	2	0	17	67	0	0	0	158
Harnischia complex sp. 3	1	0	0	0	0	1	0	5	24	31
Harnischia complex sp. 4	0	0	0	0	0	0	0	0	11	11
Harnischia complex sp. 5	0	0	0	1	0	0	0	0	0	1
Labrundinia sp. 1	0	9	10	5	0	2	48	53	32	159
Labrundinia sp. 2	0	0	6	0	0	0	0	0	0	6
Labrundinia sp. 3	0	2	1	0	0	0	0	1	6	10
Labrundinia sp. 4	0	0	0	0	0	0	1	0	1	2
Labrundinia sp. 5	0	0	0	0	0	0	0	0	1	1
Labrundinia sp. 6	0	0	1	0	0	0	0	2	4	7
Larsia sp. 1	0	1	0	0	0	0	0	0	1	2
Larsia sp. 2	0	2	0	0	0	0	0	0	0	2
Microchironomus sp. 1	0	0	0	0	0	0	0	1	0	1
Monopelopia sp. 1	0	0	0	0	0	0	2	0	0	2
Monopelopia sp. 2	0	2	0	0	0	0	0	0	0	2
Nanocladius sp. 1	176	0	46	19	6	2	3	74	136	462
Nanocladius sp. 2	1005	1	49	15	441	373	0	56	7	1947
Nanocladius sp. 3	0	0	2	4	0	0	0	0	0	6
Nilotanypus cf. *kansensis*	0	0	0	0	0	0	0	2	2	4
Nilotanypus sp. 1	67	0	1	0	22	9	0	2	0	101
Nilothauma cf. *reissi*	0	2	3	0	0	1	0	0	0	6
Nilothauma sp. 1	57	0	2	0	1	25	0	0	0	85
Nilothauma sp. 2	7	0	0	0	0	0	0	0	0	7
Nilothauma sp. 3	3	0	0	0	0	5	0	0	0	8

Species	Laguna Barra del Colorado	Laguna Cuatro	Laguna del Tortuguero	Laguna Jalova	Río Parismina	Río Pacuare	Estero Negro	Río Estrella	Río Bananito	Total
Nilothauma sp. 4	0	0	0	0	0	0	0	1	0	1
Onconeura sp. 1	11	0	0	0	0	31	0	29	3	74
Onconeura sp. 2	1	0	0	0	0	6	0	4	2	13
Onconeura sp. 3	10	0	0	1	3	29	0	16	0	59
Orthocladiinae sp. 1	0	0	0	0	0	0	0	0	1	1
Orthocladiinae sp. 2	0	0	0	0	0	0	0	1	0	1
Orthocladiinae sp. 3	0	0	0	0	0	1	0	0	0	1
Orthocladius sp. 1	1	0	0	0	0	0	0	0	0	1
Orthocladius sp. 2	0	0	0	0	0	0	0	1	0	1
Orthocladius sp. 3	0	0	0	0	0	0	0	1	0	1
Parachironomus sp. 1	0	1	1	1	0	0	0	4	0	7
Parachironomus sp. 2	1	12	3	0	0	1	0	1	0	18
Parachironomus sp. 3	0	0	1	0	0	0	0	12	5	18
Parachironomus sp. 4	0	0	0	0	0	0	0	1	0	1
Parachironomus sp. 5	0	0	1	0	0	0	0	0	0	1
Parachironomus sp. 6	0	0	8	0	0	0	0	0	0	8
Parachironomus sp. 7	0	0	0	0	0	0	0	2	0	2
Parachironomus sp. 8	0	26	0	0	0	0	0	0	0	26
Parachironomus sp. 9	0	0	0	0	0	0	0	0	1	1
Parakiefferiella sp. 1	0	0	0	0	0	0	0	0	1	1
Parakiefferiella sp. 2	0	0	0	0	0	0	0	0	1	1
Paralauterborniella sp. 1	26	0	3	156	11	3	0	0	0	199
Paralauterborniella sp. 2	0	0	17	4	11	0	0	0	0	32
Parametriocnemus sp. 1	0	0	0	0	0	2	0	1	0	3
Parametriocnemus sp. 2	0	0	0	0	0	3	0	7	1	11
Paratanytarsus sp. 1	0	0	0	0	0	0	0	0	8	8
Pentaneura sp. 1	0	0	0	0	0	0	0	1	0	1
Polypedilum sp. 1	0	26	0	0	0	0	28	0	0	54
Polypedilum sp. 10	0	0	0	0	0	0	0	11	2	13
Polypedilum sp. 11	0	0	0	0	0	0	0	21	2	23
Polypedilum sp. 12	0	0	0	0	0	1	0	6	3	10
Polypedilum sp. 13	3	0	1	5	0	7	0	6	11	33
Polypedilum sp. 14	0	20	1	0	0	0	6	1	0	28
Polypedilum sp. 15	0	0	2	1	0	1	0	3	6	13
Polypedilum sp. 16	1	36	26	4	0	4	0	7	14	92
Polypedilum sp. 17	9	2	9	43	1	2	1	0	0	67
Polypedilum sp. 18	0	0	2	1	0	0	0	0	0	3

Species	Laguna Barra del Colorado	Laguna Cuatro	Laguna del Tortuguero	Laguna Jalova	Río Parismina	Río Pacuare	Estero Negro	Río Estrella	Río Bananito	Total
Polypedilum sp. 19	0	0	0	0	0	1	0	1	0	2
Polypedilum sp. 2	4	0	33	1	7	18	0	13	1	77
Polypedilum sp. 3	0	0	0	0	0	3	14	40	212	269
Polypedilum sp. 4	21	0	0	0	9	321	0	0	0	351
Polypedilum sp. 5	1	41	71	13	1	22	238	216	162	765
Polypedilum sp. 6	0	0	0	0	0	0	0	290	51	341
Polypedilum sp. 7	0	0	0	0	0	0	0	1	1	2
Polypedilum sp. 8	1	0	0	2	0	0	0	1	2	6
Polypedilum sp. 9	0	0	0	1	0	0	0	9	44	54
Procladius sp. 1	0	0	6	0	0	0	0	0	0	6
Pseudochironomus sp. 1	5	0	7	2	0	0	0	0	0	14
Pseudochironomus sp. 2	1	0	0	0	0	0	0	106	53	160
Pseudochironomus sp. 3	0	0	0	0	0	0	0	14	0	14
Pseudochironomus sp. 4	0	0	0	0	0	0	0	6	21	27
Pseudosmittia trilobata	0	1	0	0	0	0	0	0	0	1
Rheotanytarsus sp. 1	81	0	26	2	2	16	0	1	0	128
Rheotanytarsus sp. 2	0	0	0	0	45	124	0	15	55	239
Robackia cf. *claviger*	124	0	0	0	14	95	0	3	0	236
Robackia sp. 1	42	0	0	0	4	16	0	0	0	62
Saetheria sp. 1	0	0	0	0	0	103	0	0	0	103
Saetheria sp. 2	1	0	0	0	0	1	0	1	0	3
Saetheria sp. 3	6	0	0	0	0	4	0	137	10	157
Stempellina sp. 1	0	0	3	0	0	0	0	0	0	3
Stempellinella sp. 1	0	0	0	0	0	0	0	0	17	17
Stempellinella sp. 2	3	0	2	0	0	0	0	10	2	17
Stenochironomus sp. 1	1	0	2	0	0	0	0	2	2	7
Stenochironomus sp. 2	4	0	1	2	2	8	0	2	3	22
Stenochironomus sp. 3	1	0	2	5	0	0	0	0	0	8
Stenochironomus sp. 4	0	5	2	0	0	0	0	0	0	7
Stenochironomus sp. 5	1	0	0	3	0	0	0	0	0	4
Stenochironomus sp. 6	0	1	0	0	0	0	0	0	0	1
Stenochironomus sp. 7	0	0	0	0	0	0	1	0	0	1
Stenochironomus sp. 8	4	0	0	0	0	0	0	0	0	4
Stenochironomus sp. 9	1	0	0	0	6	14	0	4	13	38
Stictochironomus sp. 1	0	0	0	0	0	1	0	35	17	53
Stictocladius sp. 1	0	0	0	0	0	0	0	7	0	7
Sublettea sp. 1	0	0	0	0	1	0	0	3	0	4

Species	Laguna Barra del Colorado	Laguna Cuatro	Laguna del Tortuguero	Laguna Jalova	Río Parismina	Río Pacuare	Estero Negro	Río Estrella	Río Bananito	Total
Tanypus sp. 1	0	0	0	0	0	2	0	0	0	2
Tanytarsus sp. 1	0	3	0	0	0	4	1	483	4	495
Tanytarsus sp. 10	0	0	0	0	0	0	0	0	1	1
Tanytarsus sp. 11	0	0	0	0	0	0	0	1	2	3
Tanytarsus sp. 12	0	0	0	2	0	0	0	0	2	4
Tanytarsus sp. 13	0	0	1	0	0	0	0	3	1	5
Tanytarsus sp. 14	0	11	0	0	0	0	0	4	0	15
Tanytarsus sp. 15	0	1	4	0	0	0	0	1	1	7
Tanytarsus sp. 16	0	2	0	3	0	0	0	0	0	5
Tanytarsus sp. 17	0	0	3	0	0	0	0	0	0	3
Tanytarsus sp. 18	0	0	0	0	0	0	37	6	23	66
Tanytarsus sp. 19	0	71	0	0	0	0	0	17	0	88
Tanytarsus sp. 2	0	3	1299	16	0	9	2	93	25	1447
Tanytarsus sp. 20	0	186	0	0	0	0	3	45	0	234
Tanytarsus sp. 21	0	0	0	0	0	0	0	1	0	1
Tanytarsus sp. 22	0	0	0	0	0	0	4	0	0	4
Tanytarsus sp. 23	1	0	0	0	0	0	0	0	0	1
Tanytarsus sp. 24	0	0	0	0	0	0	0	0	4	4
Tanytarsus sp. 3	9	0	11	10	0	1	6	10	7	54
Tanytarsus sp. 4	1	0	0	0	0	1	0	46	227	275
Tanytarsus sp. 5	11	0	21	2	0	3	0	9	76	122
Tanytarsus sp. 6	0	0	1	0	0	0	0	12	36	49
Tanytarsus sp. 7	1	10	5	9	0	4	1	45	362	437
Tanytarsus sp. 8	5	2	46	21	0	0	0	14	5	93
Tanytarsus sp. 9	0	0	0	0	0	0	0	0	1	1
Thienemanniella sp. 1	8	0	8	7	1	10	0	1	3	38
Thienemanniella sp. 2	0	0	0	0	0	0	0	2	0	2
Thienemanniella sp. 3	1	0	0	0	7	6	0	6	2	22
Thienemanniella sp. 4	4	0	0	0	12	6	0	10	19	51
Thienemanniella sp. 5	1	0	0	0	7	20	0	8	12	48
Thienemanniella sp. 6	0	0	0	0	0	1	0	0	0	1
Thienemanniella sp. 7	0	0	0	1	0	0	0	0	1	2
Thienemanniella sp. 8	0	0	0	0	0	0	0	2	1	3
Ubatubaneura sp. 1	19	0	1	0	0	0	0	0	0	20
Ubatubaneura sp. 2	3	0	0	0	0	0	0	3	2	8
Ubatubaneura sp. 3	0	0	0	0	0	1	0	0	0	1
Ubatubaneura sp. 4	0	0	0	0	0	0	0	0	1	1

Species	Laguna Barra del Colorado	Laguna Cuatro	Laguna del Tortuguero	Laguna Jalova	Río Parismina	Río Pacuare	Estero Negro	Río Estrella	Río Bananito	Total
Xenochironomus cf. *xenolabis*	1	1	0	0	0	0	0	0	0	2
Xestochironomus sp. 1	1	0	0	1	1	1	0	1	3	8
Xestochironomus sp. 2	0	0	2	0	1	3	0	12	0	18
Xestochironomus sp. 3	0	0	0	0	0	0	0	1	0	1
Xestochironomus sp. 4	0	1	0	1	0	0	0	0	0	2
Zavreliella sp. 1	0	10	9	1	0	1	0	0	0	21
Zavreliella sp. 2	0	3	1	0	0	0	0	0	0	4
Zavrelimyia sp. 1	0	0	0	0	0	0	0	0	2	2
Total	**2620**	**788**	**2043**	**1309**	**1051**	**2104**	**423**	**3620**	**3113**	**17071**

Appendix F: Chironomidae assemblage metrics employed for IBI in estuaries of the Caribbean coast of Costa Rica.

Metric Type	Metric Name	Metric Calculation Description	Response to Disturbance	Theoretical range
Richness	Total Species Richness	Total number of species	Decrease	0-∞
Richness	Margalef's Diversity Index	Margalef's Diversity Index score is based on species richness	Decrease	0-16
Relative Richness	% Tolerance	Relative richness (%) of specimens in sample with tolerance values greater than 7, using Southeast USA-derived tolerance values	Increase	0-100
Relative Richness	% Intolerance	Relative richness (%) of specimens in sample with tolerance values less than 3, using Southeast USA-derived tolerance values	Decrease	0-100
Relative Abundance	Berger-Parker Index of Dominance	Relative abundance of dominant species	Increase	0-8
Relative Abundance	% Orthocladiinae	Relative abundance (%) of Orthocladiinae	Decrease	0-100
Relative Abundance	% Chironomini	Relative abundance (%) of Chironomini	Increase	0-100
Relative Abundance	% Tanytarsini	Relative abundance (%) of Tanytarsini	Decrease	0-100
Biotic Index	Hilsenhoff's Biotic Index	Abundance weighted average of each genus using Southeast USA-derived tolerance values.	Increase	0-10
Diversity	Shannon's Diversity Index	Shannon's Diversity Index score is based on species richness and evenness	Decrease	0-4

Appendix G: Thermocycling conditions for PCR optimization.

Program 1				**Program 2**			
Step	**Temp. (°C)**	**Time (s)**	**No. of cycles**	**Steps**	**Temp. (°C)**	**Time (s)**	**No. of cycles**
Initial denaturation	95	240	1	Initial denaturation	95	300	1
Denaturation	94	30		Denaturation	94	30	
Annealing	48	30	40	Annealing	45	30	5
Extension	72	60		Extension	72	60	
Final extension	72	480	1	Denaturation	94	30	35
Hold	4	∞	1	Annealing	51	30	
				Extension	72	60	
				Final extension	72	480	1
				Hold	4	∞	1

Appendix H: Examined and DNA barcoded Chironomidae specimens from Norway. Catalog # refers to number in the insect collection of the NTNU University Museum. Note - If a specimen was not sequenced or did not have a high-quality sequence, then it will not have an GenBank Accession number.

Sample ID	Catalog #	Species Name	Author	Country	Municipality	Site	Latitude	Longitude	Elevation (m)	Collection Date	GenBank Accession
PK-186-8	146575	*Chironomus* sp. 1PK		Norway	Trondheim	Lake Lian	63.40300	10.31800	4	31-Aug-14	KT248890
PK-186-9	146576	*Chironomus* sp. 1PK		Norway	Trondheim	Lake Lian	63.40300	10.31800	4	31-Aug-14	KT248889
PK-186-10	146577	*Chironomus* cf. *tenuistylus*	Brundin, 1949	Norway	Trondheim	Lake Lian	63.40300	10.31800	4	31-Aug-14	
PK-186-11	146578	*Chironomus* cf. *tenuistylus*	Brundin, 1949	Norway	Trondheim	Lake Lian	63.40300	10.31800	4	31-Aug-14	KT248896
PK-186-12	146579	*Chironomus* cf. *tenuistylus*	Brundin, 1949	Norway	Trondheim	Lake Lian	63.40300	10.31800	4	31-Aug-14	
PK-186-13	146580	*Chironomus* cf. *tenuistylus*	Brundin, 1949	Norway	Trondheim	Lake Lian	63.40300	10.31800	4	31-Aug-14	KT248897
PK-186-14	146581	*Chironomus* cf. *tenuistylus*	Brundin, 1949	Norway	Trondheim	Lake Lian	63.40300	10.31800	4	31-Aug-14	KT248894
PK-194-1	146605	*Demicryptochironomus* sp. 1PK		Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	17-Sep-14	
PK-191-5	146603	*Polypedilum* sp. 1PK		Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	5-Sep-14	
PK-194-2	146604	*Polypedilum* sp. 1PK		Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	17-Sep-14	
PK-192-19	146681	*Micropsectra* sp. 1PK		Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	12-Sep-14	
PK-192-21	146683	*Micropsectra* sp. 1PK		Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	12-Sep-14	
PK-192-22	146684	*Micropsectra* sp. 1PK		Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	12-Sep-14	
PK-192-23	146685	*Micropsectra* sp. 1PK		Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	12-Sep-14	
PK-192-24	146686	*Micropsectra* sp. 1PK		Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	12-Sep-14	
PK-192-17	146679	*Micropsectra logani*	(Johannsen, 1928)	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	12-Sep-14	KT248910
PK-192-18	146680	*Micropsectra logani*	(Johannsen, 1928)	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	12-Sep-14	KT248909
PK-192-20	146682	*Micropsectra logani*	(Johannsen, 1928)	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	12-Sep-14	KT248908
PK-190-33	146695	*Micropsectra* sp. 1PK		Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	5-Sep-14	
PK-190-34	146696	*Micropsectra* sp. 1PK		Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	5-Sep-14	
PK-190-35	146697	*Micropsectra* sp. 1PK		Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	5-Sep-14	
PK-190-37	146699	*Micropsectra* sp. 1PK		Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	5-Sep-14	
PK-191-17	146667	*Micropsectra* sp. 1PK		Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	5-Sep-14	
PK-191-18	146668	*Micropsectra* sp. 1PK		Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	5-Sep-14	
PK-188-1	146647	*Micropsectra logani*	(Johannsen, 1928)	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	5-Sep-14	KT248906
PK-188-2	146648	*Micropsectra logani*	(Johannsen, 1928)	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	5-Sep-14	KT248907
PK-189-2	146650	*Micropsectra logani*	(Johannsen, 1928)	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	5-Sep-14	KT248919
PK-190-36	146654	*Micropsectra logani*	(Johannsen, 1928)	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	5-Sep-14	KT248918
PK-190-38	146656	*Micropsectra logani*	(Johannsen, 1928)	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	5-Sep-14	KT248917
PK-189-1	146649	*Micropsectra sp. 5ES*		Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	5-Sep-14	KT248905
PK-186-2	146551	*Tanytarsus aculeatus*	Brundin, 1949	Norway	Trondheim	Lake Lian	63.40300	10.31800	4	31-Aug-14	
PK-186-4	146553	*Tanytarsus aculeatus*	Brundin, 1949	Norway	Trondheim	Lake Lian	63.40300	10.31800	4	31-Aug-14	
PK-190-30	146608	*Potthastia gaedii*	(Meigen, 1838)	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	5-Sep-14	
PK-191-8	146611	*Potthastia gaedii*	(Meigen, 1838)	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	5-Sep-14	
PK-190-26	146571	*Corynoneura* sp. 1PK		Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	5-Sep-14	
PK-190-27	146572	*Corynoneura sp.15ES*		Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	5-Sep-14	KT248899
PK-190-15	146560	*Thienemanniella* sp. 1PK		Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	5-Sep-14	
PK-190-16	146561	*Thienemanniella* sp. 1PK		Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	5-Sep-14	
PK-190-17	146562	*Thienemanniella* sp. 1PK		Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	5-Sep-14	
PK-190-18	146563	*Thienemanniella* sp. 1PK		Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	5-Sep-14	
PK-190-19	146564	*Thienemanniella* sp. 1PK		Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	5-Sep-14	
PK-190-25	146570	*Thienemanniella* sp. 1PK		Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	5-Sep-14	
PK-191-4	146574	*Thienemanniella* sp. 1PK		Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	5-Sep-14	
PK-191-3	146573	*Thienemanniella* sp. 1PK		Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	5-Sep-14	
PK-188-10	147007	*Cricotopus* sp. 1PK		Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	5-Sep-14	

Sample ID	Catalog #	Species Name	Author	Country	Municipality	Site	Latitude	Longitude	Elevation (m)	Collection Date	GenBank Accession
PK-188-12	147009	*Cricotopus* sp. 1PK		Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	5-Sep-14	
PK-188-13	147021	*Cricotopus* sp. 1PK		Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	5-Sep-14	
PK-188-14	147022	*Cricotopus* sp. 1PK		Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	5-Sep-14	
PK-188-15	147023	*Cricotopus* sp. 1PK		Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	5-Sep-14	
PK-188-8	147016	*Cricotopus* sp. 1PK		Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	5-Sep-14	
PK-188-9	147017	*Cricotopus* sp. 1PK		Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	5-Sep-14	
PK-189-3	147000	*Cricotopus* sp. 1PK		Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	5-Sep-14	
PK-189-4	147001	*Cricotopus* sp. 1PK		Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	5-Sep-14	
PK-189-6	147003	*Cricotopus* sp. 1PK		Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	5-Sep-14	
PK-189-7	147004	*Cricotopus* sp. 1PK		Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	5-Sep-14	
PK-189-8	147005	*Cricotopus* cf. *septentrionalis*	Hirvenoja, 1973	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	5-Sep-14	KT248902
PK-189-9	147006	*Cricotopus* sp. 1PK		Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	5-Sep-14	
PK-188-11	147008	*Cricotopus* cf. *septentrionalis*	Hirvenoja, 1973	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	5-Sep-14	KT248900
PK-189-10	147007	*Cricotopus* cf. *septentrionalis*	Hirvenoja, 1973	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	5-Sep-14	KT248901
PK-189-5	147002	*Cricotopus* cf. *septentrionalis*	Hirvenoja, 1973	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	5-Sep-14	KT248903
PK-190-103	146946	*Cricotopus* cf. *similis*	Goetghebuer, 1921	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	5-Sep-14	
PK-190-104	146947	*Cricotopus* cf. *similis*	Goetghebuer, 1921	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	5-Sep-14	
PK-190-105	146948	*Cricotopus* cf. *similis*	Goetghebuer, 1921	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	5-Sep-14	
PK-190-106	146949	*Cricotopus* cf. *similis*	Goetghebuer, 1921	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	5-Sep-14	
PK-190-107	146950	*Cricotopus* cf. *similis*	Goetghebuer, 1921	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	5-Sep-14	
PK-190-108	146951	*Cricotopus* cf. *similis*	Goetghebuer, 1921	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	5-Sep-14	
PK-190-109	146952	*Cricotopus* cf. *similis*	Goetghebuer, 1921	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	5-Sep-14	
PK-190-110	146953	*Cricotopus* cf. *similis*	Goetghebuer, 1921	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	5-Sep-14	
PK-190-111	146954	*Cricotopus* cf. *similis*	Goetghebuer, 1921	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	5-Sep-14	
PK-190-112	146955	*Cricotopus* cf. *similis*	Goetghebuer, 1921	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	5-Sep-14	
PK-190-124	146967	*Cricotopus* cf. *similis*	Goetghebuer, 1921	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	5-Sep-14	
PK-190-125	146968	*Cricotopus* cf. *similis*	Goetghebuer, 1921	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	5-Sep-14	
PK-190-126	146969	*Cricotopus* cf. *similis*	Goetghebuer, 1921	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	5-Sep-14	
PK-190-127	146970	*Cricotopus* cf. *similis*	Goetghebuer, 1921	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	5-Sep-14	
PK-190-128	146971	*Cricotopus* cf. *similis*	Goetghebuer, 1921	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	5-Sep-14	
PK-190-129	146972	*Cricotopus* cf. *similis*	Goetghebuer, 1921	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	5-Sep-14	
PK-190-130	146973	*Cricotopus* cf. *similis*	Goetghebuer, 1921	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	5-Sep-14	
PK-190-131	146974	*Cricotopus* cf. *similis*	Goetghebuer, 1921	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	5-Sep-14	
PK-190-140	146983	*Cricotopus* cf. *similis*	Goetghebuer, 1921	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	5-Sep-14	
PK-190-141	146984	*Cricotopus* cf. *similis*	Goetghebuer, 1921	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	5-Sep-14	
PK-190-63	146846	*Eukiefferiella*		Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	5-Sep-14	
PK-190-64	146847	*Eukiefferiella*		Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	5-Sep-14	
PK-190-65	146848	*Eukiefferiella*		Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	5-Sep-14	
PK-190-66	146849	*Eukiefferiella*		Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	5-Sep-14	
PK-190-67	146850	*Eukiefferiella*		Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	5-Sep-14	
PK-190-68	146851	*Eukiefferiella*		Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	5-Sep-14	
PK-190-69	146852	*Eukiefferiella*		Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	5-Sep-14	
PK-190-70	146853	*Eukiefferiella*		Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	5-Sep-14	
PK-190-71	146854	*Eukiefferiella*		Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	5-Sep-14	
PK-190-72	146855	*Eukiefferiella*		Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	5-Sep-14	
PK-190-83	146866	*Eukiefferiella*		Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	5-Sep-14	
PK-190-84	146867	*Eukiefferiella*		Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	5-Sep-14	
PK-190-85	146868	*Eukiefferiella*		Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	5-Sep-14	
PK-190-86	146869	*Eukiefferiella*		Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	5-Sep-14	
PK-190-87	146870	*Eukiefferiella*		Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	5-Sep-14	
PK-190-88	146871	*Eukiefferiella*		Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	5-Sep-14	
PK-190-89	146872	*Eukiefferiella*		Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	5-Sep-14	
PK-190-90	146873	*Eukiefferiella*		Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	5-Sep-14	
PK-190-91	146874	*Eukiefferiella*		Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	5-Sep-14	
PK-190-92	146875	*Eukiefferiella*		Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	5-Sep-14	
PK-194-50	147056	*Eukiefferiella*		Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	17-Sep-14	
PK-194-51	147057	*Eukiefferiella*		Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	17-Sep-14	

Sample ID	Catalog #	Species Name	Author	Country	Municipality	Site	Latitude	Longitude	Elevation (m)	Collection Date	GenBank Accession
PK-194-52	147058	*Eukiefferiella*		Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	17-Sep-14	
PK-194-53	147059	*Eukiefferiella*		Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	17-Sep-14	
PK-194-54	147060	*Eukiefferiella*		Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	17-Sep-14	
PK-194-55	147061	*Eukiefferiella*		Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	17-Sep-14	
PK-194-56	147062	*Eukiefferiella*		Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	17-Sep-14	
PK-194-57	147063	*Eukiefferiella*		Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	17-Sep-14	
PK-194-58	147064	*Eukiefferiella*		Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	17-Sep-14	
PK-194-59	147065	*Eukiefferiella*		Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	17-Sep-14	
PK-194-70	147076	*Eukiefferiella*		Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	17-Sep-14	
PK-194-71	147077	*Eukiefferiella*		Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	17-Sep-14	
PK-194-72	147078	*Eukiefferiella*		Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	17-Sep-14	
PK-194-73	147079	*Eukiefferiella*		Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	17-Sep-14	
PK-194-74	147080	*Eukiefferiella*		Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	17-Sep-14	
PK-194-75	147081	*Eukiefferiella*		Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	17-Sep-14	
PK-194-76	147082	*Eukiefferiella*		Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	17-Sep-14	
PK-194-77	147083	*Eukiefferiella*		Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	17-Sep-14	
PK-194-78	147084	*Eukiefferiella*		Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	17-Sep-14	
PK-194-79	147085	*Eukiefferiella*		Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	17-Sep-14	
PK-192-154	146892	*Orthocladius*		Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	12-Sep-14	
PK-192-155	146893	*Orthocladius*		Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	12-Sep-14	KT248921
PK-192-152	146890	*Orthocladius oblidens*	(Walker, 1856)	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	12-Sep-14	
PK-192-153	146891	*Orthocladius oblidens*	(Walker, 1856)	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	12-Sep-14	
PK-192-156	146894	*Orthocladius oblidens*	(Walker, 1856)	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	12-Sep-14	
PK-192-157	146895	*Orthocladius oblidens*	(Walker, 1856)	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	12-Sep-14	
PK-192-168	146906	*Orthocladius oblidens*	(Walker, 1856)	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	12-Sep-14	
PK-192-169	146907	*Orthocladius oblidens*	(Walker, 1856)	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	12-Sep-14	
PK-192-170	146908	*Orthocladius oblidens*	(Walker, 1856)	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	12-Sep-14	
PK-192-171	146909	*Orthocladius oblidens*	(Walker, 1856)	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	12-Sep-14	
PK-192-182	146920	*Orthocladius oblidens*	(Walker, 1856)	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	12-Sep-14	
PK-192-183	146921	*Orthocladius oblidens*	(Walker, 1856)	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	12-Sep-14	
PK-192-184	146922	*Orthocladius oblidens*	(Walker, 1856)	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	12-Sep-14	
PK-192-185	146923	*Orthocladius oblidens*	(Walker, 1856)	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	12-Sep-14	
PK-192-186	146924	*Orthocladius oblidens*	(Walker, 1856)	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	12-Sep-14	
PK-192-187	146925	*Orthocladius oblidens*	(Walker, 1856)	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	12-Sep-14	
PK-192-188	146926	*Orthocladius oblidens*	(Walker, 1856)	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	12-Sep-14	
PK-192-189	146927	*Orthocladius oblidens*	(Walker, 1856)	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	12-Sep-14	
PK-192-190	146928	*Orthocladius oblidens*	(Walker, 1856)	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	12-Sep-14	
PK-192-191	146929	*Orthocladius oblidens*	(Walker, 1856)	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	12-Sep-14	KT248923
PK-192-107	146775	*Tvetenia calvescens*	(Edwards, 1929)	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	12-Sep-14	
PK-192-108	146776	*Tvetenia calvescens*	(Edwards, 1929)	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	12-Sep-14	KT248932
PK-192-109	146777	*Tvetenia calvescens*	(Edwards, 1929)	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	12-Sep-14	
PK-192-110	146778	*Tvetenia calvescens*	(Edwards, 1929)	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	12-Sep-14	
PK-192-111	146779	*Tvetenia calvescens*	(Edwards, 1929)	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	12-Sep-14	
PK-192-112	146780	*Tvetenia calvescens*	(Edwards, 1929)	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	12-Sep-14	
PK-192-113	146781	*Tvetenia calvescens*	(Edwards, 1929)	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	12-Sep-14	
PK-192-114	146782	*Tvetenia calvescens*	(Edwards, 1929)	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	12-Sep-14	
PK-192-115	146783	*Tvetenia calvescens*	(Edwards, 1929)	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	12-Sep-14	KT248933
PK-192-116	146784	*Tvetenia calvescens*	(Edwards, 1929)	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	12-Sep-14	KT248931
PK-192-127	146795	*Tvetenia calvescens*	(Edwards, 1929)	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	12-Sep-14	
PK-192-128	146796	*Tvetenia calvescens*	(Edwards, 1929)	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	12-Sep-14	KT248935
PK-192-129	146797	*Tvetenia calvescens*	(Edwards, 1929)	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	12-Sep-14	
PK-192-130	146798	*Tvetenia calvescens*	(Edwards, 1929)	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	12-Sep-14	
PK-192-131	146799	*Tvetenia calvescens*	(Edwards, 1929)	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	12-Sep-14	
PK-192-132	146800	*Tvetenia calvescens*	(Edwards, 1929)	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	12-Sep-14	
PK-192-133	146801	*Tvetenia calvescens*	(Edwards, 1929)	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	12-Sep-14	

Sample ID	Catalog #	Species Name	Author	Country	Municipality	Site	Latitude	Longitude	Elevation (m)	Collection Date	GenBank Accession
PK-192-134	146802	*Tvetenia calvescens*	(Edwards, 1929)	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	12-Sep-14	
PK-192-135	146803	*Tvetenia calvescens*	(Edwards, 1929)	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	12-Sep-14	
PK-192-136	146804	*Tvetenia calvescens*	(Edwards, 1929)	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	12-Sep-14	
PK-192-38	146706	*Prodiamesa olivacea*	(Meigen, 1818)	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	12-Sep-14	
PK-192-39	146707	*Prodiamesa olivacea*	(Meigen, 1818)	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	12-Sep-14	
PK-192-40	146708	*Prodiamesa olivacea*	(Meigen, 1818)	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	12-Sep-14	
PK-192-41	146709	*Prodiamesa olivacea*	(Meigen, 1818)	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	12-Sep-14	
PK-192-42	146710	*Prodiamesa olivacea*	(Meigen, 1818)	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	12-Sep-14	
PK-192-43	146711	*Prodiamesa olivacea*	(Meigen, 1818)	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	12-Sep-14	
PK-192-44	146712	*Prodiamesa olivacea*	(Meigen, 1818)	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	12-Sep-14	
PK-192-45	146713	*Prodiamesa olivacea*	(Meigen, 1818)	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	12-Sep-14	
PK-192-46	146714	*Prodiamesa olivacea*	(Meigen, 1818)	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	12-Sep-14	
PK-192-47	146715	*Prodiamesa olivacea*	(Meigen, 1818)	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	12-Sep-14	
PK-192-68	146736	*Prodiamesa olivacea*	(Meigen, 1818)	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	12-Sep-14	
PK-192-69	146737	*Prodiamesa olivacea*	(Meigen, 1818)	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	12-Sep-14	
PK-192-70	146738	*Prodiamesa olivacea*	(Meigen, 1818)	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	12-Sep-14	KT248930
PK-192-71	146739	*Prodiamesa olivacea*	(Meigen, 1818)	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	12-Sep-14	
PK-192-72	146740	*Prodiamesa olivacea*	(Meigen, 1818)	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	12-Sep-14	
PK-192-73	146741	*Prodiamesa olivacea*	(Meigen, 1818)	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	12-Sep-14	
PK-192-74	146742	*Prodiamesa olivacea*	(Meigen, 1818)	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	12-Sep-14	
PK-192-75	146743	*Prodiamesa olivacea*	(Meigen, 1818)	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	12-Sep-14	
PK-192-76	146744	*Prodiamesa olivacea*	(Meigen, 1818)	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	12-Sep-14	
PK-192-77	146745	*Prodiamesa olivacea*	(Meigen, 1818)	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	12-Sep-14	
PK-192-7	146627	*Rheopelopia maculipennis*	(Zetterstedt, 1838)	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	12-Sep-14	
PK-192-8	146628	*Rheopelopia maculipennis*	(Zetterstedt, 1838)	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	12-Sep-14	
PK-192-9	146629	*Rheopelopia maculipennis*	(Zetterstedt, 1838)	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	12-Sep-14	
PK-194-10	146630	*Rheopelopia maculipennis*	(Zetterstedt, 1838)	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	17-Sep-14	
PK-194-11	146631	*Rheopelopia maculipennis*	(Zetterstedt, 1838)	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	17-Sep-14	
PK-194-12	146632	*Rheopelopia maculipennis*	(Zetterstedt, 1838)	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	17-Sep-14	
PK-194-8	146641	*Rheopelopia maculipennis*	(Zetterstedt, 1838)	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	17-Sep-14	
PK-194-9	146642	*Rheopelopia maculipennis*	(Zetterstedt, 1838)	Norway	Trondheim	Nidelva	63.42900	10.37900	221.8	17-Sep-14	
PK-186-36	146618	*Procladius signatus*	(Zetterstedt, 1850)	Norway	Trondheim	Lake Lian	63.40300	10.31800	4	31-Aug-14	
PK-186-35	146619	*Procladius signatus*	(Zetterstedt, 1850)	Norway	Trondheim	Lake Lian	63.40300	10.31800	4	31-Aug-14	KT248928