Regional Operational Plan SF.2A.2013.02

Evaluation of Environmental DNA Techniques for the Detection of Invasive Northern Pike

by

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and

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May 2013

Alaska Department of Fish and Game **Divisions of Sport Fish and Commercial Fisheries**

Symbols and Abbreviations

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Weights and measures (metric)

centimeter	cm
deciliter	dL
gram	g
hectare	ha
kilogram	kg
kilometer	km
liter	L
meter	m
milliliter	mL
millimeter	mm

Weights and measures (English)

cubic feet per second	ft^3/s
foot	ft
gallon	gal
inch	in
mile	mi
nautical mile	nmi
ounce	oz
pound	lb
quart	qt
yard	yd

Time and temperature

day	d
degrees Celsius	°C
degrees Fahrenheit	°F
degrees kelvin	K
hour	h
minute	min
second	s

Physics and chemistry

all atomic symbols	
alternating current	AC
ampere	A
calorie	cal
direct current	DC
hertz	Hz
horsepower	hp
hydrogen ion activity (negative log of)	pH
parts per million	ppm
parts per thousand	ppt, ‰
volts	V
watts	W

General

Alaska Administrative Code	AAC
all commonly accepted abbreviations	e.g., Mr., Mrs., AM, PM, etc.
all commonly accepted professional titles	e.g., Dr., Ph.D., R.N., etc.
at	@
compass directions:	
east	E
north	N
south	S
west	W
copyright	©
corporate suffixes:	
Company	Co.
Corporation	Corp.
Incorporated	Inc.
Limited	Ltd.
District of Columbia	D.C.
et alii (and others)	et al.
et cetera (and so forth)	etc.
exempli gratia (for example)	e.g.
Federal Information Code	FIC
id est (that is)	i.e.
latitude or longitude	lat. or long.
monetary symbols (U.S.)	$, ¢
months (tables and figures): first three letters	Jan,...,Dec
registered trademark	®
trademark	™
United States (adjective)	U.S.
United States of America (noun)	USA
U.S.C.	United States Code
U.S. state	use two-letter abbreviations (e.g., AK, WA)

Mathematics, statistics

all standard mathematical signs, symbols and abbreviations	
alternate hypothesis	H_A
base of natural logarithm	e
catch per unit effort	CPUE
coefficient of variation	CV
common test statistics	(F, t, χ^2, etc.)
confidence interval	CI
correlation coefficient (multiple)	R
correlation coefficient (simple)	r
covariance	cov
degree (angular)	°
degrees of freedom	df
expected value	E
greater than	>
greater than or equal to	≥
harvest per unit effort	HPUE
less than	<
less than or equal to	≤
logarithm (natural)	ln
logarithm (base 10)	log
logarithm (specify base)	$\log_2$, etc.
minute (angular)	'
not significant	NS
null hypothesis	H_O
percent	%
probability	P
probability of a type I error (rejection of the null hypothesis when true)	α
probability of a type II error (acceptance of the null hypothesis when false)	β
second (angular)	"
standard deviation	SD
standard error	SE
variance	
population	Var
sample	var

REGIONAL OPERATIONAL PLAN SF.2A.2013.02

EVALUATION OF ENVIRONMENTAL DNA TECHNIQUES FOR THE DETECTION OF INVASIVE NORTHERN PIKE

by

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Alaska Department of Fish and Game
Division of Sport Fish

May 2013

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Project leader(s): Kristine Dunker and Robert Massengill

Division of Sport Fish , Region II, Soldotna Office

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Approval

Title	Name	Signature	Date
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PURPOSE

Organisms naturally shed cells or cell fragments containing their DNA into the environment. Such DNA is referred to as environmental DNA (eDNA). eDNA from aquatic organism can be collected from water samples. This study will contract the development of genetic markers for northern pike *Esox lucius* and utilize those markers to detect northern pike eDNA in water samples using a genetic laboratory technique called polymerase chain reaction (PCR). Testing will determine how sensitive to detection northern pike eDNA is under controlled aquaria and field conditions (i.e. volume of water sample, density of northern pike in the waterbody, length of northern pike presence (live and post-mortem), sample distance from northern pike, etc). eDNA holds promise as a fish survey tool that has the benefits of being species-specific, faster, more cost-effective, and more sensitive to detecting low-density fish populations compared with traditional fish survey methods (i.e. gillnetting, traps or electrofishing). If demonstrated to be an effective fish survey tool, eDNA detection techniques would improve the Department's ability to evaluate the success of invasive northern pike eradication and control efforts and in assessing northern pike distribution in Southcentral Alaska.

BACKGROUND

Northern pike do not naturally occur in Southcentral Alaska although they are native to many areas north and west of the Alaska Range and in a limited area near Yakutat (Mecklenburg et al. 2002). Northern pike were first illegally introduced to Southcentral Alaska in the Susitna River drainage at Bulchitna Lake in the 1950's (ADFG 2007). The proliferation and expansion of northern pike from this initial introduction and additional illegal stockings has resulted in northern pike establishment throughout much of the Susitna River drainage, some lakes and creeks of the Knik Arm and Western Cook Inlet, at least three waterbodies in the Anchorage area and eighteen waterbodies on the Kenai Peninsula. To date, of the nineteen Kenai Peninsula waterbodies where northern pike were confirmed to be present, only eleven lakes and Soldotna Creek are currently known to support pike populations (Figure 1).

Northern pike are valuable sport and subsistence fish where they are native, but they are also top-level predators of fish and other aquatic fauna. When introduced to waters outside of their native range, northern pike can harm native fish populations. Invasive northern pike have caused the extirpation of three-spined stickleback *Gasterosteous aculeatus* from Prator Lake in south-central Alaska (Pantankar, 2006). In Southcentral Alaska, northern pike prey heavily on rearing salmonids and, in some cases, have been implicated in the decline of local salmon runs (Rutz 1996 and 1999) or complete localized loss native fish (McKinley 2013). A recent study documenting northern pike stomach contents in the Alexander drainage suggests northern pike predation offers a plausible explanation for the dramatic decline in salmonid abundance (Sepulveda et al. 2013). Similarly, other northern Cook Inlet waters have experienced drastic reductions of their resident fish populations such as Fish Creek of Kroto slough, Fish Lake Creek of the Yentna River and Fish Creek of the Nancy Lake canoe system (ADFG 2007).

On the Kenai Peninsula, native salmonid populations have been decimated by northern pike predation in places such as Stormy Lake within the Swanson River drainage and from several lakes within the Soldotna Creek drainage (ADFG 2012; McKinley 2013). Because of the ecological and economic impacts associated with their introductions, northern pike are an invasive species in Southcentral Alaska.

In 2008, the Alaska Department of Fish and Game (ADFG) began a program to eradicate invasive northern pike from select Southcentral waterbodies. To date, northern pike have been removed from five Kenai Peninsula lakes and two Anchorage lakes. Rotenone (a fish piscicide) was used to remove northern pike from three Kenai Peninsula lakes and two Anchorage lakes and gillnets were used to eradicate northern pike from two Kenai Peninsula lakes containing very small northern pike populations (N<30).

A continuing challenge for ADFG has been the inability to definitively ascertain where invasive northern pike are present, particularly in areas where they may be present in very low abundance such as after an eradication attempt or in a recently infested waterbody. Typically, resource agencies use nets, traps, visual observations or electrofishing to conduct fish surveys. A survey effort capable of detecting a small northern pike population can require an immense commitment of staff and financial resources. Most survey methods are reliable indicators of species presence only if the target organism is present in moderate-to-high abundance (Magnuson 1994). As an example, ADFG estimated that the gillnetting effort required to detect at least one northern pike, with a 90% probability of detection, from a 400 surface-acre lake with only four northern pike present was >16,000 net soak hours utilizing variable mesh nets that are 120 feet in length and six feet in depth (ADFG 2012). Another concern with netting and electrofishing surveys are that both have the potential to harm non-target species.

Technological advances in molecular techniques such as amplification of DNA through PCR using simpler and more cost-effective means of sequencing have made the analysis of eDNA more widespread (Toothman 2008, Beja-Periera 2009, Darling 2011). The application of noninvasive genetic techniques is now relatively common in the identification of an organism's DNA through analysis of tissue, fluids or excrement (Beja-Pereira et al. 2009). More recently, noninvasive genetic techniques have been used to detect the presence of rare or invasive aquatic organisms by analyzing water samples for eDNA (Ficetola 2008, Thomsen 2011, Jerde 2011). The basic premise is that organisms leave behind DNA as they pass through the environment from hair, feathers, shed epidermal cells, feces, urine, gametes or other sloughed tissues (Jerde 20011, Lydolph 2005 and Haile 2009). For aquatic organisms, eDNA suspended in the water can be collected in water samples wherein the DNA is filtered, preserved, extracted, amplified, then analyzed to determine if genetic markers of the target species are present (Ficetola 2008, Jerde 2011). Potentially, eDNA methodology could be a faster, cost-effective and more reliable tool for verifying the presence or absence of invasive northern pike, particularly where they are present in very low abundance. Another significant benefit of noninvasive genetic sampling is that it poses no risk of harm to the study species (Beja-Pereira 2009) therefore there is no harm to non-target species.

This study will contract the development of northern pike genetic markers and utilize them in evaluating the detectability of northern pike eDNA from water samples collected from aquaria and field trials. Field trials will be used to assess the detectability of northern pike eDNA infiled conditions and its persistence following carcass deposition. This study will also apply eDNA detection methods to sample tributaries for northern pike presence, including a lake where the Department recently attempted to eradicate invasive northern pike. Using a combination of approaches, results from this study will allow us to make inferences on the detectability of northern pike eDNA while manipulating variables such as density of northern pike in the waterbody, sample volume size, distance sampling occurs from caged pike and persistence of eDNA over time post-mortem.

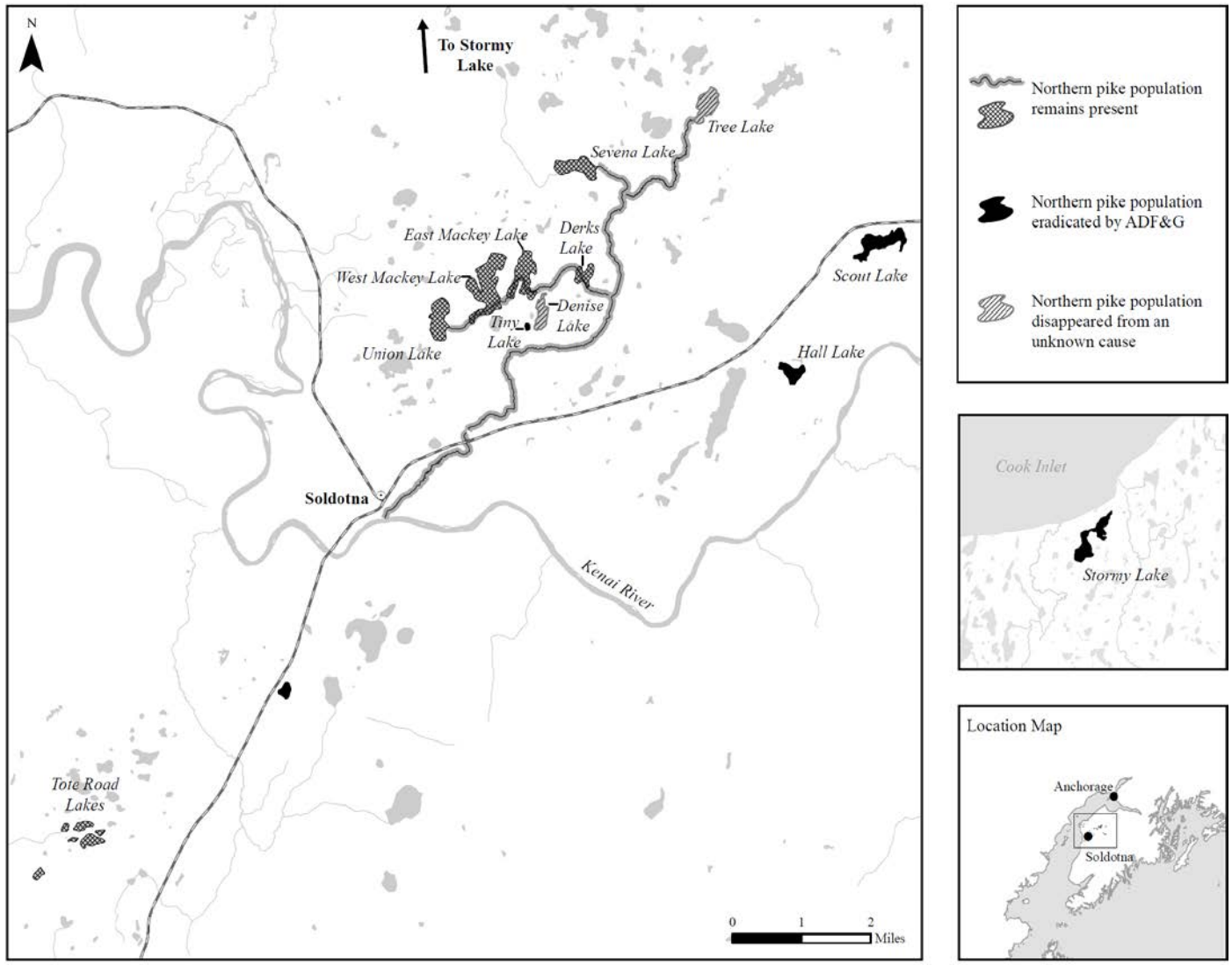

Figure 1. Status of Kenai Peninsula waterbodies where self-sustaining populations of northern pike have occurred.

OBJECTIVES

The research objectives for the northern pike eDNA study in 2013 will be to:

1. Estimate the probability of detecting northern pike eDNA from different water sample volumes collected from four replicate aquaria stocked with a known density of northern pike such that the probability of detection is within 18 percentage points 90% of the time.[1,2]
2. Estimate the probability of detecting northern pike eDNA in field trial lakes all stocked with a similar density of northern pike (~0.032 kilograms of northern pike per acre-foot) by collecting and analyzing twenty-four water samples from each lake after the northern pike have been present for one week such that the probability of detection is within 10 percentage points 90% of the time.[3]
3. Estimate the probability of detecting eDNA from live northern pike at three sampling distances (one, ten and forty meters) by collecting and analyzing eight water samples samples from each distance from each of three similarly-sized lakes such that the probability of detection is within 17 percentage points 90% of the time.[3]
4. Determine how long eDNA from decomposing northern pike remains detectable in three similarly-sized study lakes wherein all will be stocked with northern pike carcasses in the test cages at a density of ~1.0 kilograms/acre-foot by collecting and analyzing twenty-four water samples from the sampling distances listed in objective #3 from each lake one week after the carcass stocking, then biweekly for four weeks, then monthly thereafter until northern pike eDNA is undetectable.[3]

[1] This objective will help us determine the minimum water sample volume needed to detect northern pike eDNA during field trials that yields a probability of detection >0.5 using PCR analysis.

[2] Each aquarium will hold 75 liters of water stocked with one to three northern pike totaling ~181 grams in weight

[3] Volume of water sample will be determined based on aquaria trial results.

Tasks

1. Determine whether northern pike eDNA is detectable in a single field trial lake stocked with an extremely low density of caged northern pike (0.002 kilograms of pike per acre-foot) by collecting and analyzing twenty-four water samples from the lake after the northern pike have been present for one week.
2. Contract a genetics laboratory to develop species-specific genetic markers and analysis protocols for northern pike eDNA detection purposes by June 30, 2013.
3. Contract a laboratory to perform the qPCR analysis of the eDNA samples generated from this study by June 30, 2013.
4. Analyze water samples for northern pike eDNA collected from the Soldotna Creek drainage, Alexander Creek drainage[4], Moose River drainage, Swanson River drainage and Russian River drainage to assess whether northern pike eDNA detection results align with traditional sampling and harvest reporting information.
5. Collect and analyze Stormy Lake water samples for northern pike eDNA before and after a rotenone treatment to investigate the effectiveness of this method for evaluation of northern pike eradication projects.

[4] The Alexander Creek drainage is outside the Kenai Peninsula but due to its prevalent northern pike population will serve as a false-negative control for northern pike DNA detection.

METHODS

EXPERIMENTAL AND SAMPLING DESIGN

Following the contracted development of the northern pike genetic marker(s) and PCR protocols that are fundamental to this project, we will use aquaria trials to determine whether the the genetic markers can be detected from aquaria water samples, and if so, determine the minimum water sample volume that yields a positive northern pike eDNA detection with a probability >0.5.

For the aquaria trials, we will collect and test for eDNA using six different volumes of water collected from each of four replicate 75-liter aquaria each stocked with approximately 181 grams of northern pike. The sample volumes will range from 0.50 µl as the smallest to 50 ml as the largest. We will also have two controls which will be aquaria set up identical to the others but not stocked with northern pike.

For each sample volume to be tested, we will collect five samples per aquarium (4 stocked aquariums X 5 samples per volume/aquarium = 20 water samples per volume). This sample size will allow estimating the detection probabilities within 18 percentage points of the true values 90% of the time, assuming binomial distribution and the 'worst-case' scenario probability of 0.5. We will test six different volumes of water yielding a grand total of 180 water samples including the control samples (30 samples/aquarium X 6 aquaria = 180 water samples)

Once the aquaria trial samples are collected and the samples analyzed, results will determine our minimum sample volumes appropriate for the field trial component of this study. (see *Sample Volume Determination* on page 10).

During the field trials, twenty-four water samples will be collected from each of four lakes during each sampling event. Three of the lakes will serve as replicates and will be stocked with a similar low density of caged northern pike (0.032 kg/acre-foot); the fourth lake will be stocked with an extremely low density of northern pike (0.002 kg/acre-foot). The volume of the water samples collected during the field trials will be determined by the aquaria trial results, but the sample volume selected should theoretically provide a probability of detection of >0.5 per sample for the lake stocked with the lowest pike density (Appendix A1). Therefore, the probability of detecting northern pike eDNA in at least one of the twenty-four samples would theoretically be ≥ 0.999 (i.e.$(1.0 - 0.5^{\wedge 24})$).

For Objective 2, collecting 24 samples from each of the three replicate lakes will allow estimating detection probability of pike eDNA within 10 percentage points 90 % of the time, assuming binomial distribution, p=0.5, no lake-effect, and no distance-effect.

All water samples collected during the field trials will be systematically collected at predetermined distances from the cages holding northern pike. Doing so will help us assess how distance from the caged fish affects eDNA detection success.

For Objective 3, collecting 24 samples from each of the three replicate lakes will allow estimating detection probability for each sampling distance within 17 percentage points 90 % of the time, assuming binomial distribution, p=0.5, and no lake-effect.

Additional experimental and sample design methodology is discussed in more detail later in this section.

NORTHERN PIKE COLLECTION

Northern pike used in the aquaria and field study trials will be collected from the Kenai Peninsula. Because the field trials will occur within the Soldotna Creek Drainage, an attempt will be made to collect northern pike from within that drainage. Lakes in the drainage known to support northern pike populations include: Union Lake, East and West Mackey Lakes, Derks Lake and Sevena Lake. Union Lake and Derks Lake probably offer the best opportunity to collect enough northern pike based on recent netting results in 2011 and 2012. If capturing enough northern pike for study purposes is not attained in the Soldotna Creek drainage, additional northern pike will be collected from the Tote Road lakes located about five miles south of Soldotna (Figure 1).

Northern pike collected for the field study component will be captured as soon as open water permits during late April or early May of 2013. This period is within the spawning season. Only males >250mm in total length will be retained for study purposes so there is no risk that both sexes are available for reproduction should fish escape. Identification of sex will be confirmed by release of gametes upon manual manipulation of the pelvic area. Females and those fish where sex cannot be confirmed will be killed and used later for carcass stocking of the field study lakes or donated for food and educational purposes.

A variety of gear types may be employed for northern pike capture that include gillnets, hook and line, electrofishing, hoop nets fyke nets and backpack electrofishing. Gillnetting is likely to be the primary gear used. To reduce injury and stress to gillnet caught fish, gillnets will be monitored continuously. A gillnetting strategy that has worked well is to position a gillnet parallel to a highly vegetated shoreline using a boat and then drive fish hiding along the shoreline into the net by walking the shoreline while probing the shoreline with a stick or paddle.

All fish collected for aquaria trials will be held in a net pen in the lake until they are transferred to the study trial location. All transported fish will be placed in insulated aerated containers during transport and water temperatures will not exceed 10 °C. All study fish will be measured for fork length (FL) and weighed to the nearest gram prior to inclusion in the study.

AQUARIA TRIALS

Aquaria Stocking

Intuitively, the concentration of an aquatic organism's eDNA in water is related to its abundance or density although attempts to link relative eDNA strength to abundance is not well established (Thomsen 2011, Jerde 2011). We realize there are many factors that could affect the availability or persistence of eDNA and therefore our ability to detect it. These factors include: time of day, growth status of the species, disposition of the DNA, presence of non-target DNA, degradation from: temperature, sunlight, toxins, microbial actions, enzymatic action, low pH and hydrolysis among others (Hawkins 2012, Dejean 2011, Telechea 2009). However, there is evidence that residence time of detectable eDNA ranges from days to several weeks (Matsui 2001, Dejean 2011 (as reported in Hawkins 2012) and Thomsen 2012). For this study we won't know whether northern pike density is directly linked to northern pike eDNA concentration or whether northern pike eDNA is evenly distributed throughout a waterbody.

For study purposes we define an extremely low northern pike density as: 181 grams (0.4 lbs) of northern pike per 100 acre-feet of water. This density is in alignment for waterbodies where a

small number of individuals survive an eradication attempt or in waterbodies recently invaded by northern pike. A density on a similar scale was observed in Anchorage at Cheney Lake (174 acre-feet) in 2011 when a small number of northern pike were apparently reintroduced after a 2008 rotenone treatment. A public report of a northern pike observed at Cheney Lake in 2011 led the Department to intensively gillnet the lake throughout the winter of 2011/2012 until ice-out. In total, four northern pike of similar size (~450mm each) were removed and none have been observed or reported in the lake since. Based on length, each fish was estimated to weigh 1.3 pounds (Willis 1989). The density of northern pike in Cheney Lake in 2011 was estimated to be 3.0 pounds of northern pike per 100 acre-feet of water. The length of these same fish one year prior (2010) was estimated to be ~210 mm and is inferred by the growth rate of northern pike observed in another Southcentral Alaska lake (ADFG undated). Likewise the 2010 density of northern pike in Cheney Lake was estimated to have been ~0.3 pounds of northern pike per 100 acre-feet.

The density of northern pike (grams of northern pike per liter of water) stocked in the aquaria will be unavoidably high compared to densities found in most field situations because it is impractical to obtain and manage aquaria large enough to mimic natural densities. For example, one small 181-gram northern pike in a 75-liter aquarium has an equivalent fish density of 2,977 kilograms of northern pike per acre-foot which is several orders of magnitude greater than densities in some local lakes based on recent removal estimates (Massengill 2011). Nonetheless, the target stocking density for the aquaria trials of 181 grams/75 liters is one of practicality.

Aquaria Setup and Maintenance

The aquaria for the study trials will consist of six insulated chest coolers. Four aquariums will be used to hold northern pike and two will be used as controls with no northern pike added. Prior to filling, all aquaria equipment will be sterilized by triple-rinsing with a bleach solution (1 part bleach: 10 parts water) then triple rinsed with untreated well water. Each aquarium will then be filled with 75 liters of well water. The aquaria water will be aerated so dissolved oxygen does not fall below 7.0 ml/g per liter which is the concentration where juvenile growth can be impeded (Inskip 1982). A water chiller will be used in each aquarium to prevent the temperature from exceeding 12 °C, which is at the high end of northern pike spawning suitability (Inskip 1982). The chilled water should slow microbial and algae growth. The length of time the northern pike will be held in each aquarium (three days) will likely be too brief for nitrogen compounds to reach lethal concentrations.

No filtration system or chemical additives will be used as the effects of either to DNA persistence and availability are unknown. If a northern pike dies in an aquarium during the three day holding period it will be replaced as quickly as possible with a new live fish of similar size. The northern pike will not be fed during the aquaria trials. Just prior to stocking the aqauria and immediately following the aquaria trials basic water quality data will be collected from each aquarium using a Hydrolab™ Quanta that will measure temperature, specific conductance, pH and dissolved oxygen. In addition, ammonia, nitrate, nitrite and phosphate levels will be monitored daily with test strips as will temperature using individual thermometers placed in each aquarium. All northern pike will be sacrificed following the aquaria trials.

Sample Volume Determination

The primary goal of the aquaria trials is to determine the minimum water sample volume that can consistently (>50% of the time) detect northern pike eDNA so inferences can be made on the

appropriate water sample volume to collect during the field trials. A typical field water sample volume used for eDNA detection of aquatic invasive species (AIS) is one or two liters (Jerde 2011, Blankenship 2011). Some studies have had good eDNA detection success collecting as small as 15 ml samples (Ficetola 2008, Thomsen 2011), but detection success decreased in headwater areas with these smaller sample volumes.

To compensate for the relatively high concentration of northern pike and resultant eDNA expected in the aquaria water versus field study waters, we will test for eDNA using a range of much smaller sample volumes (compared to one-liter or two liter samples). By inversely reducing the aquaria water sample volume to compensate for the greater pike density stocked in the aquaria (compared to the field trials), we hope to capture similar amounts of eDNA as one-liter field samples would.

Appendix A1 provides details on how we determined the range in water sample volumes for the aquaria trials and the results are listed below in Table 1 in both microliters and milliliters.

Table 1. Range of water sample volumes given in micro liters and milliliters that will be collected to test for northern pike eDNA detections during the aquaria trials.

sample volume in microliters	sample volume in millilters
0.5	0.0005
5.0	0.005
50.0	0.05
500.0	0.5
5,000.0	5
50,000.0	50

Aquaria and Control Sampling

After northern pike have been held in aquaria for three days, the water samples listed in Table 1 will be collected from each aquarium (including the control aquariums). Five samples will be collected from each aquarium for each sample volume. A total of 180 aquaria water samples will be collected. All samples will be collected using an autoclaved graduated cylinder by inserting the cylinder several inches below the water surface to draw the sample then transferring it to an autoclaved glass centrifuge vial where samples will be preserved with 95% ethanol so that at least a 3:1 ETOH/water ratio is achieved. For very small target volumes <5.0ml, we will collect a standard 10 ml sample of aquaria water which will be diluted with well water in a sterilized container so that a new 10ml sample of this dilution will theoretically contain an equivalent amount of eDNA as the original target volume (Appendix A1 provides details for the dilutions).

Three 50 ml well water sample mixed with DNA extracted directly from a northern pike will serve as a false-negative experiential controls and three 50 ml well water samples will serve as a false-positive controls for the well water. All samples collected from the control aquaria (without any northern pike) will serve as false-positive experimental controls. Additional false-positive controls (travel blanks) will consist of collecting a sample of deionized water for each sample volume being tested (six total). All other controls and blanks will be similarly preserved

in ETOH. Data recorded for all samples will include the date, time, collector initials, aquaria identification or control identification, water sample volume and an assignment of a unique sample number. The unique sample number will be labeled on each sample vial with no other information so samples can be submitted blindly for analysis. All sample vials will be sealed individually in Whirl-Pac® bags and stored in a freezer until submitted to a laboratory for analysis.

FIELD TRIALS

Study Lake Information

Applying the minimum water sample volume needed for the study lake trials (based on results from the aquaria trials); we will attempt to detect northern pike eDNA from four small closed natural lakes stocked with northern pike. The lakes selected for the field trials are all within the Soldotna Creek drainage, a drainage which already contains invasive northern pike in some of its larger lakes and streams (Figure 1). The four lakes selected for the field trials are Denise Lake, Tiny Lake and two unnamed lakes referred to as Little Bear Lake and Gensle Lake (Figure 2, Table 2).

Each lake is currently barren of fish except threespine stickleback *Gasterosteus aculeatus.* Recent gillnetting of these lakes confirmed that northern pike are not present in any, however, northern pike did exist in Tiny Lake and Denise Lake until recently. In 2010, Denise Lake was subjected to 2,517 hours of netting effort and no pike were caught despite northern pike being confirmed there in 2003. It is unknown what caused the northern pike population in Denise Lake to disappear although the population was never believed to have been large based on anecdotal information. At Tiny Lake, northern pike were first confirmed there in 2010 but local residents have suggested they were present there for many years. ADFG removed the entire northern pike population (N<30) from Tiny Lake in 2010 using gillnets. Following the removal effort, ADFG continued to gillnet Tiny Lake under the ice during 2010/2011 for a total of 14,981 hours of under-the-ice net soak hours and no northern pike were detected.

Water samples will be collected from each field study lake before introducing caged pike and then after one week of the northern pike being present. Afterwards, all the northern pike will be sacrificed and allowed to decompose in their cages including the input of additional northern pike carcasses so that additional periodic water sampling can determine the length of time northern pike eDNA persists and is detectable post-mortem.

Table 2. Field study lake data and associated caged live northern pike stocking density targets.

Lake Name	Surface Acreage	Volume in Acre-feet	Stocking Density	Total kilograms of pike stocked	Total pounds of pike stocked
Gensle Lake	2.85	19	.032 kilograms/acre-foot	0.6	1.3
Tiny Lake	4.63	31	.032 kilograms/acre-foot	1.0	2.2
Little Bear Lake	5.5	38.5	.032 kilograms/acre-foot	1.2	2.7
Denise Lake	43	639	.002 kilograms/acre-foot	1.3	2.8
			Total	4.1	9.0

Lake volume estimates already exist for Tiny and Denise Lake but are needed for Gensle Lake and Little Bear Lake. To estimate lake volume, we anticipate collecting lake location and depth data using a Lowrance™ HDS 7 Touch depth finder/chart plotter mounted on an outboard boat. The Lowrance™ HDS 7 Touch unit will collect GPS location data by being linked to a Trimble GeoXT GPS. Depth data will be collected using a standard skimmer transducer linked to the Lowrance™ HDS 7 Touch unit. The Lowrance™ HDS 7 Touch unit simultaneously records coupled location and depth data at a user-selected "ping rate" (5 to 20 signals per second). Data can be collected automatically with the Lowrance™ HDS 7 Touch unit while boating within the lake boundaries. Typically, data collection first begins by traveling around the perimeter of the lake then continuing to travel in increasingly smaller concentric patterns until the lake is fully covered. While collecting data the spacing between boat paths will be <5 meters. All data records can then be saved by the Lowrance™ HDS 7 Touch unit to an SD media card and will be uploaded to ciBioBase for data processing. ciBioBase is a subscription-based software service provided by Contour Innovations LLC. CiBioBase is a cloud-based GIS software platform that automates data processing of Lowrance™ HDS sonar logs and creates GIS map layers of bathymetry, submerged vegetation abundance, and bottom hardness. ADFG will subscribe to ciBioBase in order to process data collected from study systems and acquire precise datasets of water volume and other companion data layers.

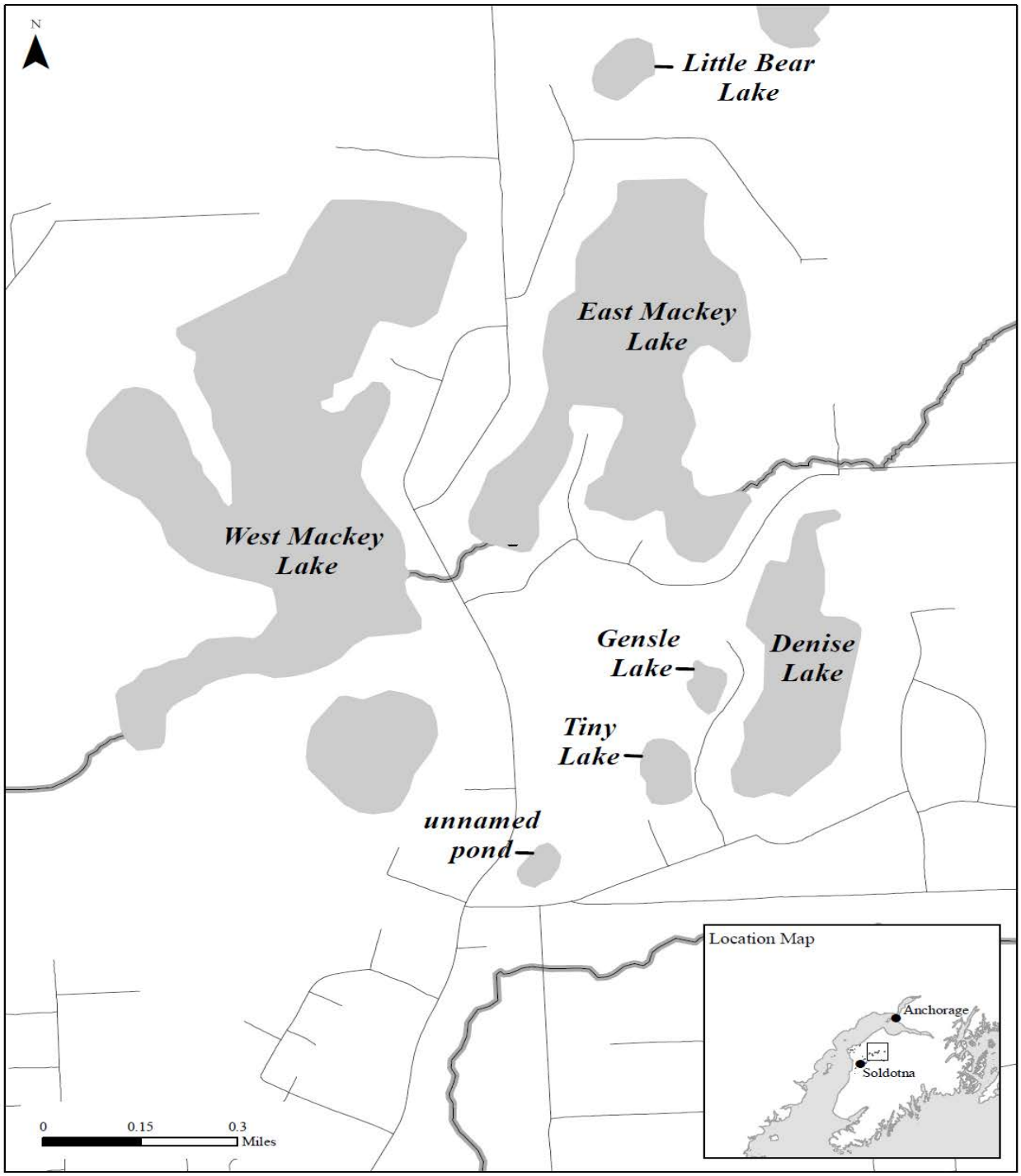

Figure 2. Locations of the Soldotna Creek drainage lakes used for the field trials.

Lake Stocking

The stocking of each study lake will be done in a manner that ensures a reproducing northern pike population is not established. All northern pike selected for stocking will be male based on expression of milt upon manual manipulation (collection will occur during the spawning season). All stocked northern pike will be placed inside cages made from heavy-duty plastic drums. The

cages will be liberally vented to allow free water exchange but the vent openings will be too narrow to allow northern pike passage. The containers will be suspended mid-column in the lakes by tethering to floating buoys and, to prevent drifting, they will also be attached to anchors or posts driven into the lake bed.

The location where each cage is placed will be recorded with a Trimble GeoXT GPS with sub-meter accuracy. The cages will be placed near shoreline habitat that is consistent with that typically utilized by northern pike (i.e. boundary of aquatic macrophyte growth) (Inskip 1982). An effort will be made to space the containers as equidistant from each other as reasonable in relation to the shoreline perimeter of the lake. Appendix A2 depicts the approximate cage placement within each study lake.

All the cages within a specific lake will be stocked with a similar weight of northern pike to more evenly distribute eDNA, as opposed to all or most of the fish being held in single cage/location. The three smallest lakes will be stocked with a density of caged live northern pike (~0.032 kilograms of northen pike per acre-foot). This density represents a very low density population and is just 5.5% of the northern pike density that actually existed in Tiny Lake (31 acre-feet lake in the Soldotna Creek drainage) where in 2010 all the northen pike were removed will gillnets and an estimated 18.14 kilograms (0.59 kg/acre-foot) of northern pike were removed. For comparison, the biomass per surface acre in Tiny Lake (4.6 surface acres) was estimated at 3.96 kilograms; in north-central Minnesota, mean biomass per surface acre of sixteen northern pike populations averaged 5.9 kilograms of northern pike per surface acre (Pierce 2012). Denise Lake, the largest of the four study lakes, will be stocked with northern pike at a much lower northern pike density (~0.002 kilograms of northern pike per acre-foot). This will allow for assessment of northern pike eDNA detectability when the density represents just four individuals (0.3 kilogram average weight) in over six hundered acre-feet of water.

For reference, the density of the northern pike at Denise Lake will be similar to individual density reported in another study that consistently detected the eDNA of frogs and salamanders using only 15 ml water samples (Thomsen 2011). Conversely, brook trout eDNA detections in a 53 acre-foot lake (volume inferred indirectly) were less successful and averaged a 25% positive detection rate despite using larger water sample volumes (one liter) and having a higher individual density of target animals (~28 brook trout/acre-foot).

All stocked northern pike will be killed after one week. All northern pike carcassess will be removed from Denise Lake but not at the remaining three study lakes (Tiny lake, Gensle Lake and Little Bear Lake) as each will be stocked with additional northern pike carcasses to attain a similar carcass density of ~1.0 kilograms of northern pike carcasses per acre-foot. The carcassess will be stocked in the same cages and locations the live northern pike were held. Placing the carcasses in cages will prevent carcass removal by scavengers and also contain the carcassess to a precise area so that assessment of eDNA detectability as a function of distance can be evaluated. In each lake, the northern pike carcasses will be evenly distributed between the four cages similar as with the live northern pike that were stocked. Water samples will be collected periodically from set distances (1, 10 and 40 meters) to assess northern pike eDNA persistence post-mortem which is explained in greater detail in the next section.

Field and Control Sampling

Just prior to stocking the study lakes with caged northen pike, four separate one-liter water samples will be collected and analyzed for eDNA from each lake (including the control lake)

to confirm that northern pike eDNA is not detectable in any lake. The four samples collected from each lake will be collected from the locations where the pike cages will be placed. Water sample collections will begin in each study lake after the caged northern pike have been held alive in them for one week.

Post-mortem water sampling of the lakes stocked with northern pike carcasses will be conducted one week following the carcass stocking, then biweekly for four weeks, then monthly thereafter until eDNA is no longer detectable.

During all lake sampling events (pre and post-mortem), twenty-four water samples will be collected from each lake including the control lake... The sampling strategy will be to collect two samples from each of three distances (1 meter, 10 meters and 40 meters) from each of the four cages in the lakes. To standardize this sampling strategy, each study lake will be divided into four quadrants for sampling. The quadrants will be created by dividing the lake into similar-sized sections using north-south and east-west axis lines that cross in the approximate center of the lake. The northern pike cages will be placed on the borders of each quadrant within ten yards of the shoreline. In each quadrant two composite water samples will be collected from each sample site (Appenidx 2). During each weekly sampling event, six water samples will be collected from each of four quadrants (3 sample distances X 2 samples per site) yielding a total of 24 samples per lake (4 quadrants X 6 samples/quadrant). All water samples will be collected no further than ten meters from the shoreline where northern pike are typically found in neighboring lakes.

Tethered buoys will be used to mark all lake sample site locations to facilitate repeatability, and these locations will also be recorded with a Trimble GeoXT GPS. The volume of each water sample will be at least one liter unless results from the aquaria trials indicate otherwise. Samples will be collected in plastic Nalgene sample bottles. Sample bottle labels will be labeled with a unique sample number. The sample number will be used to link each sample to its relevant data record that will include:

1) Lake name and northern pike stocking density (kilograms/acre-foot)
2) Lake quadrant (Norths, South East or West)
3) Sample distance from nearest cage
4) Pre or post-mortem sample status
5) Collector initials
6) Project title (northern pike eDNA study).

All water samples will be composite samples collected using a Nasco™ Swing Sampler. The swing sampler consists of a long handle that can extend to twelve feet. At one end is a clamping device that can hold a bottle in the one-liter volume range. The clamping device can be rotated between zero and ninety degrees in relation to the handle axis. Fixing the sample bottle at an angle to the handle axis will facilitate holding the sampling bottle upside down (open end facing downwards) as it enters the lake thus forming an airlock. To fill the bottle at a desired depth simply rotate the handle to break the airlock and the sample bottle will fill. To collect each composite sample, we will collect water from three different depth strata as follows: 1) within one foot of the lake surface, 2) mid-water column grab, and 3) within one foot of the lake bottom. Each discrete depth strata sample will contribute approximately one-third composite sample volume.

During each sampling period, we will collect a field blank (of the same volume used for the lake sampling) using deionized water while at each study lake. For each sampling period we will also prepare a single travel blank, using deionized water, that will accompany us while collecting field samples to examine whether we are getting false positive detections from handling or transport/shipping. Each sampling period the travel blank will be prepared in the lab and transported to and from the field in the coolers used to transport the study samples. The field blanks will be handled and transported using the same protocols as the study samples. In addition to the travel and field blanks, we will collect experimental control samples that will consist of twenty-four water samples (same volume as the study lake samples) collected from a nearby 1.3 surface-acre pond (Figure 2) where northern pike have never been detected. Four empty pike cages, identical to those used to hold northern pike in the field trial lakes, will be suspended in the control pond similar to the study lake cages.

To reduce the chance of cross-contamination of samples between study lakes or sample sites, new nitrile gloves will be worn by the collector at each sampling site. At each sample site the sample will be collected using a swing sampler that has been disinfected with a 10% bleach solution and triple rinsed with deionized water. All samples collected in the field will be chilled by packing them in ice inside an insulated cooler.

Within two days of sample collection, all samples will be filtered using a GeoTech series II peristaltic pump and 0.45 μm nitrocellulose membrane filters. After filtering, all membrane filters from unique samples will be stored in separate vials and placed into cold storage. If multiple filters are required to filter a single sample, all can be combined in a single vial. Each day samples are filtered, a control sample (same volume as the study lake samples) using deionized water called a "lab blank" will be filtered to detect if contamination is occurring during the filtering process. Details on the field water sampling, equipment decontamination, sample filtering and storage protocols are found in Appendix A3.

During the field study water sampling, basic water quality data will be collected from each study lake on every day sampling occurs using a Hydrolab Quanta to measure water temperature, specific conductance, pH and dissolved oxygen. Daily noon weather conditions will be recorded throughout the study lake sampling period to include cloud cover, temperature, dew point, humidity, barometric pressure, wind speed and direction and precipitation by accessing weather records found online at: WWW.WeatherForYou.com.

Drainage Sampling

We will collect water samples from select Kenai Peninsula drainages that encompass a range of northern pike densities based on public reports and ADFG netting data to evaluate eDNA detection success. The sampling intensity will be dependent upon funding but, at a minimum, at least 424 one-liter water samples will be collected amongst the drainages in total. The Soldotna Creek drainage (Kenai River tributary) will be sampled because it has a well established northern pike population inhabiting some of its open lakes and at least some that inhabit the mainstem of the creek. The Soldotna Creek sampling will therefore serve as a control to test whether false-negative detections are prevalent in a system with a low to moderate relative pike density. Alexander Creek in the Susitna drainage (north of the Kenai Peninsula and Anchorage) will be sampled because it supports a high relative density of northern pike and can serve as a false-negative control for eDNA detection where northern pike densities are higher than those found on the Kenai Peninsula.

The Moose River drainage will be sampled as it is considered at high risk to northern pike invasion and occasional reports of northern being caught by anglers have occurred there since the 1980's. To date there is no evidence a self-sustaining northern pike population exists in this drainage.

The Swanson River drainage will also be sampled because it has been considered at high risk for northern pike infestation. However, the only confirmed occurrence of northern pike in this drainage has been in Stormy Lake that was treated with rotenone to remove its northern pike population in 2012. Stormy Lake drains into the Swanson River near rivermile one via a small creek that averages 1-2 cfs (ADFG 2012). *See Stormy lake section below*

The Russian River drainage will be sampled as a control for false positive eDNA detections. This drainage receives an intense amount of sport fishing effort and an ADFG fish weir is operated annually just below lower Russian Lake during June through early September. To date, no northern pike have ever been observed by ADFG at the weir or reported caught by anglers in the ADFG Statewide Harvest Survey.

Most of the Kenai Peninsula drainage sampling will be in lakes (384 samples) and lake selections will target lakes that are prone to northern pike invasion due to their connectivity to known northern pike waters. Within these lakes, sampling sites will be selected based on what visually appears to be suitable northern pike habitat i.e. calm shallow waters containing abundant macrophyte growth. The desired sampling intensity for each sampled lake is one water sample per 20 surface acres (and a minimum sample size of five per lake regardless of size) but will ultimately be limited to what funding for sample processing will allow.

To assess whether flowing waters within the drainages (including the Alexander Creek) have detectable northern pike eDNA, at least ten water samples will be collected from each drainage (50 total) within their lower reach except for Alexander where the upper reach areas are known to have higher northern pike densities which will allow us to test for eDNA detections over a wider range of densities. If additional funding becomes available, a more intensive sampling effort will occur in the Swanson River and Moose River drainages as these two drainages are believed the most vulnerable to northern pike invasion and detection of northern pike in either would be highly useful information for fisheries managers. A list of the potential lake sampling locations is found in Appendix A4.

Stormy Lake Rotenone Treatment Evaluation

Stormy Lake in the Swanson River Drainage was treated with rotenone in September of 2012 to remove its northern pike population. On September 4 2012, the day before rotenone treatments in Stormy Lake began, 48 locations of the lake were sampled for northern pike eDNA. At each site, one two-liter sample and three 15 ml water samples were collected. Three field and travel blanks using deionized water were also collected at the lake. GPS coordinates were collected using a Trimble GeoXT at all sample locations to ensure repeatability of the sampling effort. The primary purpose was to determine if northern pike eDNA could be detected in Stormy Lake prior to treatment. An effort was made to sample areas of the lake that were known, based on earlier radio-telemetry data (Massengill *In Prep.*), to be occupied by northern pike. Thirty samples were collected in these known pike locations, and the remaining 18 sights were sampled representatively throughout the lake in areas where northern pike were not known to occur to investigate if pike eDNA could be detected throughout the lake or only in areas we would expect to detect it (Appendix A5). The two sample volumes, 2-L vs. 15 ml., were collected at each site

to see if differences existed in the eDNA detection rates between the large and small sample sizes. Following the sample collection, the 2-L samples were filtered and preserved in ethanol in the Soldotna Limnology lab using a GeoTech series II peristaltic pump and 0.45 µm nitrocellulose membrane filters. After filtering, all membrane filters were stored in separate vials and preserved with 95% ethanol. All 15-ml samples were fixed in the field with 35 ml of 95% ethanol and 1.5 ml of sodium acetate (Ficetola et al. 2008). All pre-treatment filtered and fixed samples were archived in a freezer (-20 °C) at the Anchorage Gene Conservation lab for future eDNA analysis. This study will resample Stormy Lake post-treatment (summer of 2013) following the same protocol as conducted pre-treatment to assess whether northern pike eDNA is detectable following the rotenone treatment and to compare those results with the results of the archived pre-treatment samples. Ultimately, this component of the project will establish whether or not eDNA will be an appropriate tool for determining the success or failure of future northern pike eradication projects.

DATA COLLECTION

All data captured during the this study will be recorded electronically in Excel spreadsheets and archived by RTS in Anchorage.

DATA ANALYSIS

Aquaria Trials

Aquaria trials will be used to estimate the probability of detecting northern pike eDNA in different water sample volumes. Four replicate aquaria will be stocked with the known equal density of northern pike and two control aquaria will be set up identically to others but not stocked with northern pike (for more details see *Experimental and Sampling Design* section). A total of 180 samples will be collected during these trials – five samples per sample volume tested by six aquaria by six sample volumes. Since individual aquariums represent random effects, we will use a generalized linear mixed model (GLMM) for binomial data to describe the relationship between DNA detection and water sample volume. In this experiment, the response variable of interest is the proportion of positive detection of northern pike eDNA. Sample volume will be set as fixed effects. Probability of detection of northern pike DNA (p) and its variance will be estimated for each sample volume using statistical software package SAS (ref) as described in Littell et al. (2006). For each sample volume, alternative hypothesis Ha: $p > 0.5$ will be tested against the null hypothesis Ho: $p \leq 0.5$ with the significance level $\alpha = 0.05$. The smallest sample volume that rejects the null hypothesis in favor of the alternative, i.e. that yields detection probability significantly greater than 0.5, will be used to come up with the equivalent minimum water sample volume to collect during the field trials (Appendix A1).

Field Trials

During the field trials, 24 water samples will be collected from each of four lakes during each sampling event. Three lakes will be replicates and stocked with a similarly low density of northern pike (0.032 kg/acre-foot); a fourth lake wil be stocked with an extremely low density of northern pike (0.002), more than an order of magnitude less than the three replicate lakes. Each lake will be divided into four similarly-sized quadrants for sampling. In each quadrant, two water samples will be collected from each of three predetermined distances from caged northern pike. For the three low density replicate lakes, we will use a generalized linear mixed model for binomial data to describe the relationship between DNA detection and pike density in the lake as

well as sampling distance. In this experiment, the response variable of interest is the proportion of positive detection of northern pike eDNA. Sampling distance represents fixed effects while lakes represent random effect. Probability of detection of northern pike DNA (p) and its variance as well as the lake-effect on the probability of detection will be estimated using statistical software package SAS (ref) as described in Littell et al. (2006). Probability of detection of northern pike eDNA for each sampling distance will also be estimated and the interaction effects of lakes and sampling distance will be evaluated.

To determine the length of time northern pike eDNA remains detectable in field trials after the completion of the live fish trials, all three low density replicate lakes will be stocked with pike carcasses (~1.0 kg/acre-foot) and sampled periodically until eDNA is no longer detectable. 24 samples will be collected from each lake during each sampling event. The detection will be deemed successful if at least one of the 24 samples in each replicate lake is positive for northern pike eDNA. Even if probability of detection drops to only 10% per sample event, collecting 24 samples will yield the probability of detection of 92% in at least one of the 24 samples.

SCHEDULE AND DELIVERABLES

Dates of events and other activities are summarized below. All research results will be compiled in a State of Alaska Fisheries data Series report.

Date(s)	Activities
February 20 to June 30, 2013	Northern Pike genetic marker development, contract a lab for sample processing if needed
April 22 to May 15, 2013	Northern pike collection, aquaria set-up, start aquaria trials, begin collecting and processing water samples
May 16, 2013	Field trial setup, begin trials, collect and process samples weekly until eDNA is not detectable
May 14 to July 15, 2013	Collect drainage/Stormy Lake samples, process samples
July 16 to August 15, 2013	Conclude field trial water sample collection and processing
November 1, 2013	Project data entered into spreadsheet
February 1, 2014	Data analysis complete
April 1, 2014	Draft report submitted to biometrician
June 1, 2014	Draft report to research supervisor

RESPONSIBILITIES

List of Personnel and Duties:

Fishery Biologist III (Tim McKinley) Oversee operational and reporting duties, assist with field activities and sampling

Fishery Biologist III (Kristine Dunker) Coauthor operational plan and FDS report, assist in all aspects of aquaria and field work including sample processing and data collection.

Biometrician III (Anton Antonovich) Provide biometric support for operational planning and FDS report, may assist with field work.

Fisheries Biologist II (Robert Massengill) Coauthor operational plan and FDS report, assist in all aspects of aquaria and field work including sample processing and data collection. Supervise technician and provide logistical support.

Fish and Wildlife technician III (Jerry Strait) Duties, assist with northern pike capture, aquaria and field set up, sampling, sample processing and data collection.

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Appendix A1. Method to determine the range of water sample volumes to collect during the northern pike eDNA aquaria trials and the water sample volume to collect during subsequent field trials.

The primary goal of the aquaria trials is to determine the minimum water sample volume that can consistently (>50% of the time) detect northern pike eDNA so inferences can be made on the appropriate water sample volume to collect during subsequent field trials. For the aquaria trials, we plan to collect water sample volumes from four replicate 75-liter aquaria each stocked with 181 grams of northern pike. A typical field water sample used for eDNA detection of aquatic invasive species (AIS) sampling is one or two liters in volume (Jerde 2011, Blankenship 2011) although at lest one some study have collected five and ten liter water samples (Goldberg 2011). Some studies have had good eDNA detection success collecting 15 ml water samples (Ficetola 2008, Thomsen 2011), but detection success decreased in headwater areas.

To compensate for the relatively high concentration of northern pike eDNA expected in the aquaria water versus the field study waters, we will test the aquaria water for eDNA using a range of smaller sample volumes (compared to one-liter field samples). By inversely reducing the aquaria water sample volumes to compenate for the greater pike densities found in the aquaria (compared to the field trials), we hope to capture somesimilar amounts of eDNA as one-liter fieldsamples would.

Here we calculate the range in water sample volumes we will need to collect for the aquaria trials where the density of northern pike is expected to be up to several orders of magnitude higher than that found in the field trials.

A northern pike density of just 0.4 pounds (181 grams) per 100 acre-feet of water appears to be a realistic density for waters recently pioneered by northern pike or where a small population survived an eradication attempt or winterkill event. This density will represent the lowest density of northern pike that we will attempt to detect eDNA from during the field trials. The field trials will test the northern pike eDNA detection sensitivity for three density levels as follows:

1) 181 grams of northern pike per $1/10^{th}$ acre-foot,
2) 181 grams of northern pike per 1 acre-foot,
3) 181 grams of northern pike per 10 acre-feet, and
4) 181 grams of northern pike per 100 acre-feet

The volume of water sample collected during the field trials will be determined by the eDNA detection results from the aquaria trials, but for this exercise, we assume the field study water sample volumes will be one-liter.

The maximum difference in density of northern pike stocked in the field trials (at Densie Lake) versus the aquaria trials will be used to determine the minimum water sample volume to collect during the aquaria trials. In addition to collecting a minimum water sample volume during the field trials, we will also collect a range of larger water sample volumes to ensure we actually detect eDNA. In theory, we expect to collect a similar amount of northern pike eDNA from the minimum aquaria water samples as with a one-liter field trial sample where pike density is lowest (181 grams of northern pike per 100 acre-feet).

To facilitate calculating the relative differences in northern pike density between the aquaria trials and the field trials, we first establish equivilant units for the different volumes discussed.

One acre-foot = 1,233,481.85 liters (ℓ)

One 20-gallon aquarium = 75ℓ

1,000,000 micro liters (µl) = 1ℓ

If an equal amount of northern pike (181 grams) were stocked in both a one-acre foot waterbody and a 75ℓ aquarium, the density of northern pike in the aquarium would be 16,466.4 times greater (1,233,481.85 ℓ ÷75 ℓ = 16,466.4).

Hypothetically, to collect a similar amount of eDNA from the aquaria water samples as that collected in one-liter field trial samples, we must reduce the aquaria sample volumes relative to their increased fish density. The four northern pike stocking densities to be tested during the field trials are listed below. For each field trial fish density level, we list the relative increase in fish density of the aquaria trials (aquaria fish density increase) followed by the calculation to adjust the aquaria water sample so that a similar amount of eDNA is captured as in the field trials. All aquaria trials will be stocked at 181 grams of northern pike per 75 liters of water.

Field trial fish density levels	Density increase over aquaria	Estimated aquaria sample volume
181 grams/1/10th acre foot	1,647	1 ℓ ÷1,6,46.6 = .00061 liters or 60.1 µl
181 grams/acre foot	16,466	1 ℓ ÷16,466.4 = .000061 liters or 60.1 µl
181 grams/10 acre-feet	164,664	1 ℓ ÷164,664 = .0000061 liters or 6.1 µl
181 grams/100 acre-feet	1,646,640	1 ℓ ÷1,646,640 = .00000061 liters or 0.61 µl

Because we don't yet know the minimum sample volume that allows for detection of northern pike eDNA in the aquaria trials, we will begin with a volume of 0.50 µl which is slightly less than the estimated smallest aquaria sample volume of 0.61 µl listed in the above table. We will then collect increasingly larger sample volumes until six samples of different volumes are collected as follows:

Sample#	Sample volume
Sample 1	= 0.50 µl
Sample 2	= 5.0 µl
Sample 3	= 50.0 µl
Sample 4	= 500.0 µl (0.5 ml)
Sample 5	= 5,000 µl (5 ml)
Sample 6	= 5,000 µl (50 ml)

The dispostion and distribution of northern pike eDNA in the aquaria during the trials will be unknown and there is concern that extracting small water volumes from the aquaria using a transfer pippette or similar device could either miss or filter particles containing eDNA due to the small pore size of the pipette. To alleviate some of these concerns, we intend to collect an equivilant amount of eDNA for each of the four smallest sample volumes (500.0 µl, 50.0 µl,5.0 and 0.5 µl) by diluting 10ml aquaria water samples with clean well water, then collecting a new 10 ml water samples from the dilutions using a graduated clylinder. By adjusting the amount of well water to mix with each 10ml aquaria water sample, we can collect a new 10 ml sample from the dilution that contains an equivilant amount of eDNA as the original water sample volume would have captured without concern that eDNA was being filtered.

The following table provides the amount (millilters, liters or gallons) of well water required to dilute a 10 ml aquaria water sample wherein the resulting dilution is capable of yielding a new 10 ml sample that contains an equivilant amount of eDNA as the original water sample desired.

Sample volume goal (ml)	Actual sample size collected from pike aquaria (ml)	Dilution factor needed to obtain an equivilant amount of DNA in a 10 ml "resample" of the dilution as that found in a "sample volume goal" listed in column B	Milliliters needed to dilute sample appropriately	Liters needed to dilute sample appropriately	Gallons needed to dilute sample appropriately
50	50	Not applicable - water sample size/pippette pore diameter is sufficent	N/A	N/A	N/A
5	5	Not applicable - water sample size/pippette pore diameter is sufficent	N/A	N/A	N/A
0.5	10	20	190	0.19	0.1
0.05	10	200	1,990	1.99	0.5
0.005	10	2000	19,990	19.99	5.3
0.0005	10	20000	199,990	199.99	53.5

Once the aquaria trials are completed and we are able to determine the minimum water sample volume goal equivilant that provides for detection of northern pike eDNA >50% of the time, we can estimate the minimum water sample volume needed for the field trials. This is calculated by multiplying the minimum aquaria water sample volume that supported detection of northern pike eDNA by the appropriate “aquaria fish density increase” found in the above table

For example, if the minimum aquaria trial water sample volume that supported detection of northern pike eDNA >50% of the time is 0.5 µl, we multiply this volume by the “aquaria density increase factor” for the field trial with the lowest fish density (181 grams (~0.2 kilograms) of fish per 100 acre-feet) to determine the field trial minimum water sample volume (Equation 1).

Equation 1 = M*A

where:

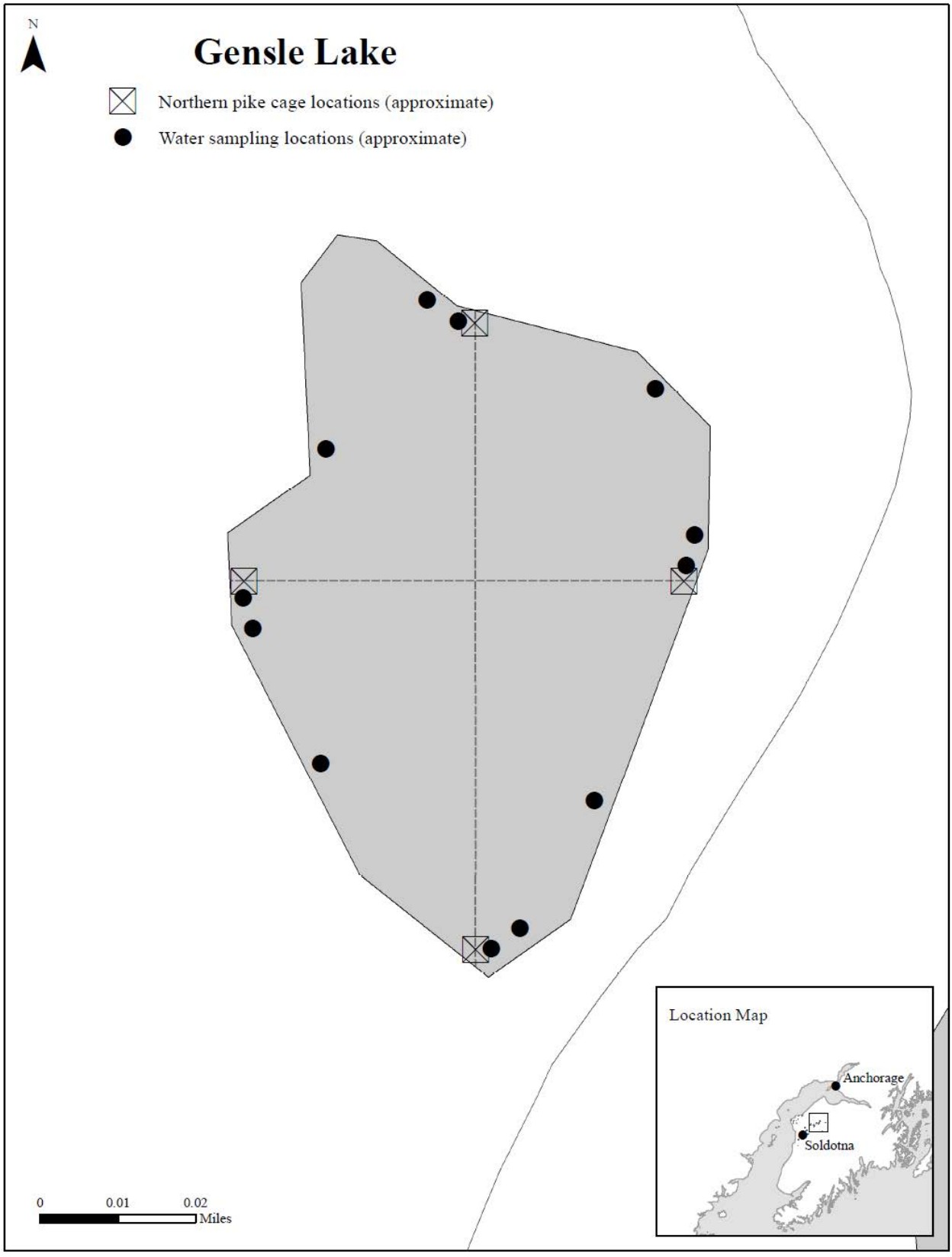
M = the minimum aquaria water sample that detects northern pike eDNA >50% of the time and,

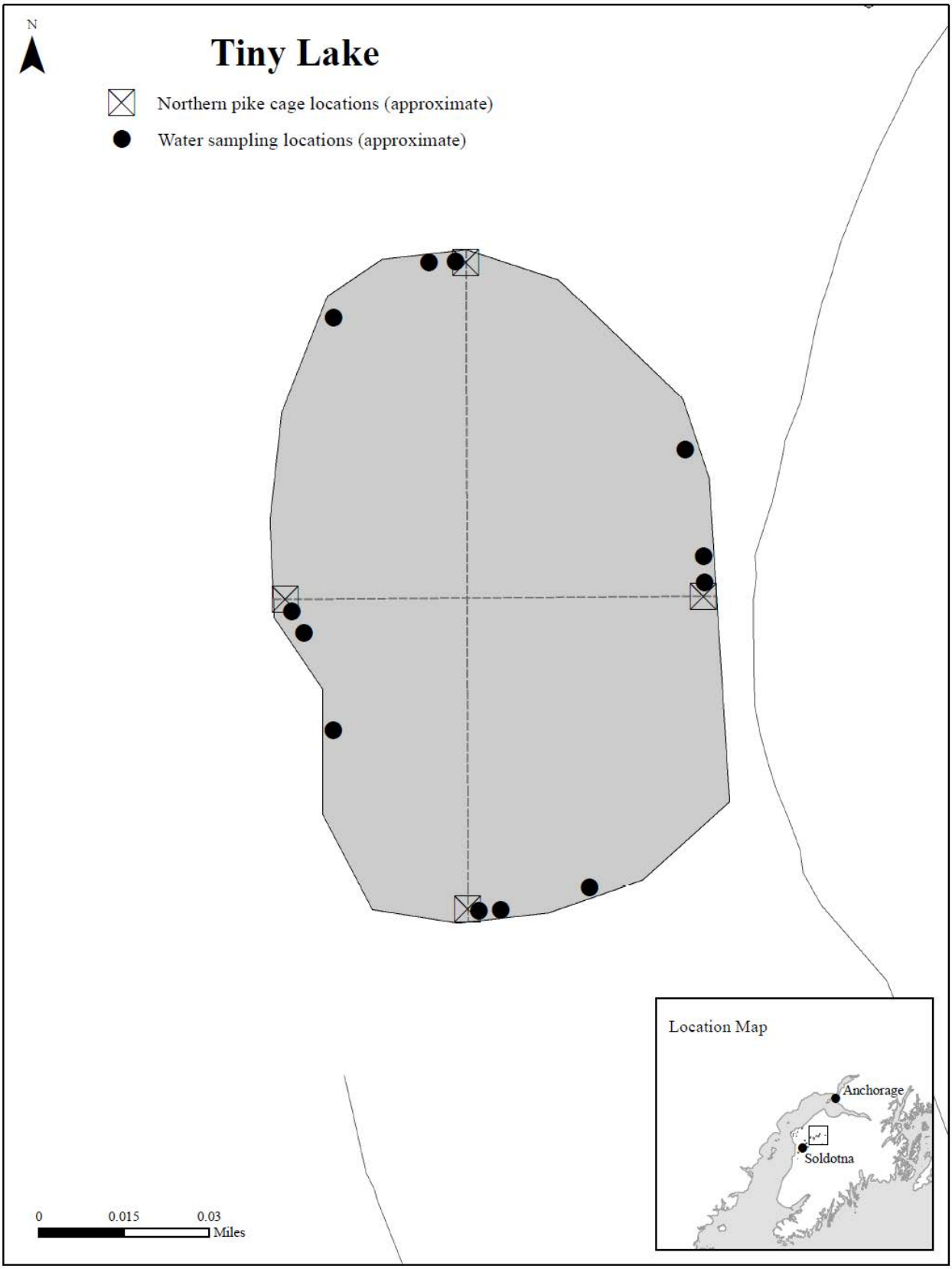
A= the “aquaria fish density increase” factor for the field trial with the lowest fish density

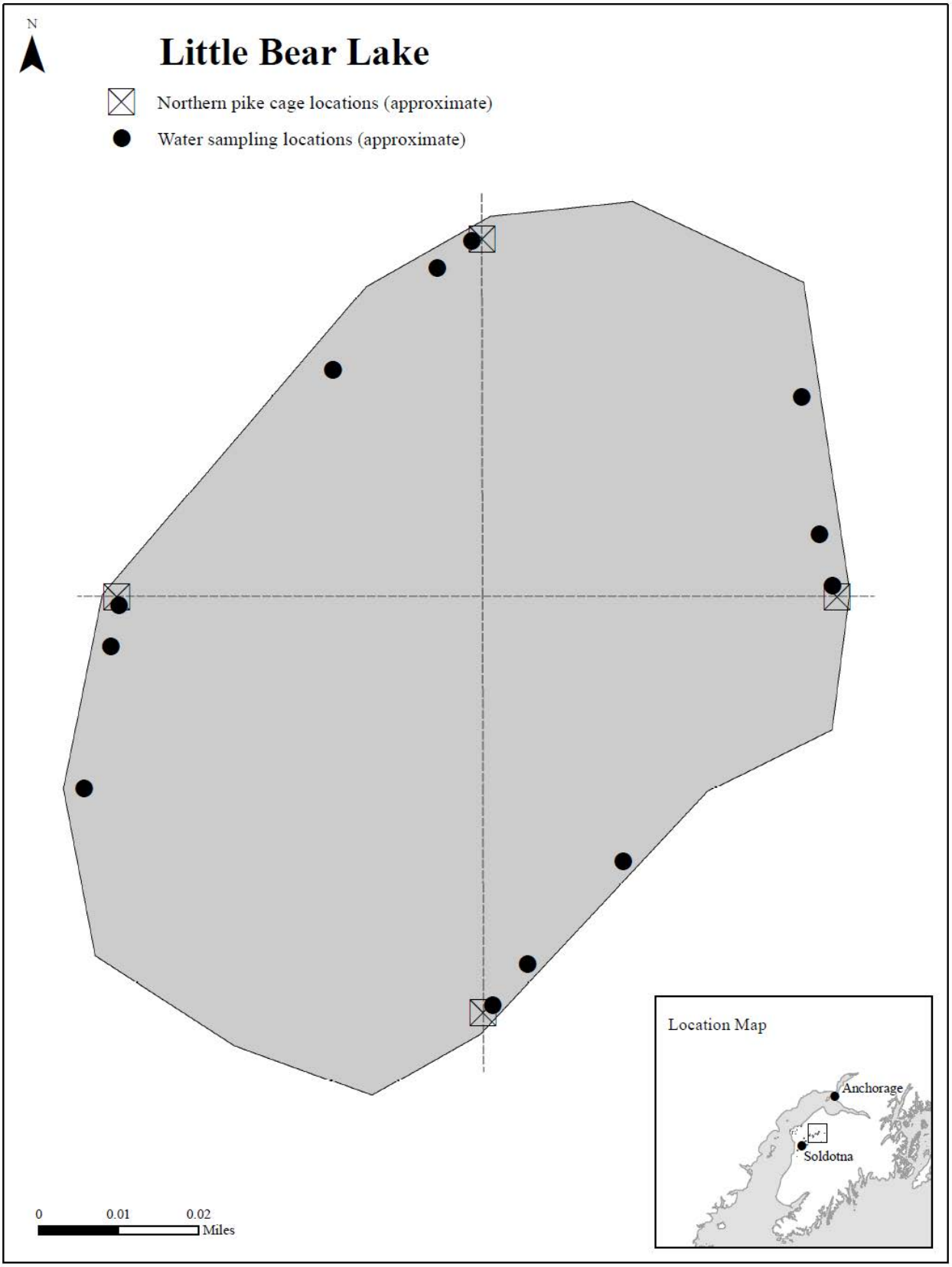
therefore,

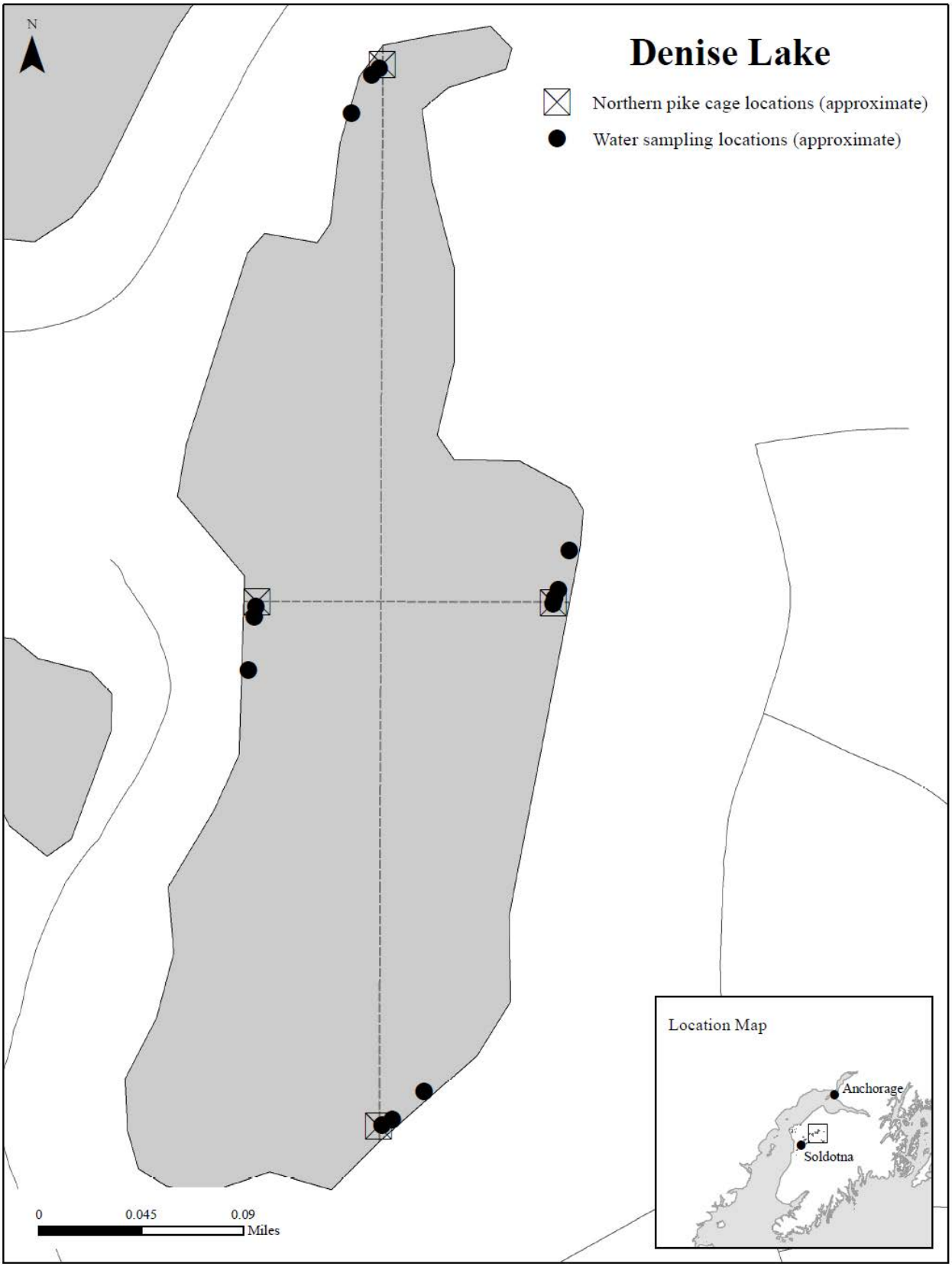
the minimum field trial water sample volume to collect if 0.5 µl aquaria samples were successful in northern pike eDNA detection is: 0.5 µl * 1,646,640 = 823,320 µl = .82 liters

Appendix A2. Maps of the four lakes used for the eDNA field trials showing approximate placement for the caged northern pike and water sampling sites.




N
Tiny Lake
Northern pike cage locations (approximate)
Water sampling locations (approximate)
Location Map
Anchorage
Soldotna
0
0.015
0.03
Miles


N
Little Bear Lake
Northern pike cage locations (approximate)
Water sampling locations (approximate)
0
0.01
0.02
Miles
Location Map
Anchorage
Soldotna


N
Denise Lake
Northern pike cage locations (approximate)
Water sampling locations (approximate)
0
0.045
0.09
Miles
Location Map
Anchorage
Soldotna

Appendix A3. Field sampling equipment decontamintaion and the filtering/storage protocol for all samples

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Field Sampling Contamination Prevention Protocol

- All samples will be collected in new plastic Nalgene bottles
- A Swing Sampler will be used to hold the sample bottle while collecting the water
- All Nalgene bottles will be placed inside of one-gallon Zip-loc™ bags after then are filled with the water sample
- The Swing Sampler must be disinfected in a 10% bleach bath then triple rinsed with deionized water bewteen all sample site locations
- Samplers must wear new nitrile gloves at each sampling site (12 sites per lake)
- Each field study lake, including the control lake, will be sampled from a boat that is either decontaminated before entering the water by rinsing the hull with a 10% bleach followed by a deionized water rinse or by committing a boat to each lake for the duration of the study so cross-contamination between lakes cannot occur via the boat as a vector.

Sample Filtering and Storage Protocol

- In the lab, cut tubing sections (both silicone and polyethylene) for all samples to be filtered, label them (#1-x) and store them in a clean ziplock bag until use.
- Assemble the GeoTech pump with clean tubing.
- Set up a tray with 50% bleach solution for sterilizing forceps and used tubing and two trays with DI water for rinsing.
- Sterilize the in-line fiter before filtering the first sample
- With a clean set of nitrile gloves and sterilized forceps, insert a membrane filter into the inline filter
    - Make sure the o-ring is centered well on the filter
- Pump water from the sample bottle.
- Once pumping is complete for a sample, carefully remove the filter with forceps.
    - Carefully fold the filter and place in a storage vial filled with 95% ethanol.
    - Label the vial and place in a separate whirpak bag. Label the outside of the whirlpack and store ina freezer.
    - Note: More than one filter may be required for each sample. If so, store all filters for each sample together in the same vial.
- After each sample, rotate forceps between the bleach solution, DI water trays, and dry paper towels. Only use forceps that have been through the entire decontamination process before removing the next filter.
    - Always change gloves between samples.
    - Always change pump tubing between samples and sterilize the used tubing.
    - Always sterilize the in-line filter between samples by soaking in a 50% bleach solution followed by double rinsing to follow using DI water.
    - Always pump one liter of DI water through the pump and in-line filter (without filter inserted) between samples.
- Do not filter samples in chronological lake order. Rather, filter the first sample collected from each lake, then filter the second sample collected from each lake, then the third and so forth to avoid potential error associated with the amount of time between sample collection and filtering.
- On each day of filtering, pour DI water into 1-L bottles and filter the same as other samples. These will serve as equipment blanks (controls) for the project.

Appendix A4. Potential Kenai Peninsula Northern Pike eDNA waterbody sampling locations by drainage, including the lake surface acres, number of water samples desired and mode of access.

Potential Northern Pike eDNA sampling locations, mode of access and number of samples				
Moose River drainage				
Drainage	Waterbody Name	Surface acerage	Number of water samples	Access[a]
Susitna River	Alexander Creek (sloughs in upper third)	N/A	10	B
	Total	0	10	
Moose River	Moose River Mainstem	N/A	10	B
Moose River	Afonsai Lake	110	6	V
Moose River	Bear lake	56	3	A
Moose River	Camp Island Lake	435	22	A
Moose River	Clam Lake	357	18	A
Moose River	Egumen Lake	105	5	V
Moose River	Grebe Lake	373	19	A
Moose River	Imeri Lake	18	1	V
Moose River	Kelly Lake	151	8	V
Moose River	Loon Lake	612	31	A
Moose River	Meadow Lake	87	4	A
Moose River	Moosehorn Lake	281	14	A
Moose River	Peterson Lake	97	5	V
Moose River	Rock Lake	355	18	A
Moose River	Swan Lake	848	42	A
Moose River	Watson Lake	58	3	V
	Total	3,943	207	
Swanson River	Swanson River Mainstem	N/A	10	B
Swanson River	Akula Lake	183	9	A
Swanson River	Campers Lake	103	5	A
Swanson River	Campfire Lake	78	4	A
Swanson River	Gene Lake	215	11	A
Swanson River	Gruska Lake	91	5	A
Swanson River	Hat Lake	36	2	A
Swanson River	Leaf Lake	62	3	A
Swanson River	Lonely lake	57	3	A
Swanson River	Lower Crane (east) Lake	35	2	A
Swanson River	McClain Lake	284	14	A
Swanson River	Pepper Lake	407	20	A
Swanson River	Rodent Lake	10	1	A
Swanson River	Stormy Lake	403	20	V
Swanson River	Upper Crane	51	3	A
Swanson River	Wild lake	64	3	A
	Total	2,079	114	
Soldotna Creek	Soldotna Creek Mainstem	N/A	10	V
Soldotna Creek	Cisca Lake	71	4	A
Soldotna Creek	Derks Lake	33	2	V
Soldotna Creek	East Mackey Lake	96	5	V
Soldotna Creek	Sevena Lake	74	4	V
Soldotna Creek	Tree Lake	64	3	A
	Total	338	27	
Russian River	Russian River Mainstem	N/A	10	V
Russian River	Lower Russian Lake	215	11	A
Russian River	Upper Russian Lake	1,100	55	A
	Total	1,315	76	
	Grand total	7,675	424	
a A = aircraft access, B = Boat, V = vehicle/walk-in access				

Appendix A5. Approximate sampling locations for northern pike eDNA in Stormy Lake collected on September 4, 2012 and to be repeated during the summer of 2013. Samples collected in high northern pike use areas of the lake are in blue (#1-30). Sampled collected in low-use areas of the lake are in turquoise (#31-48).