Transactions of the American Fisheries Society

ISSN: 0002-8487 (Print) 1548-8659 (Online) Journal homepage: http://tandfonline.com/loi/utaf20

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To cite this article: Barry P. Baldigo, Lee Ann Sporn, Scott D. George & Jacob A. Ball (2017) Efficacy of Environmental DNA to Detect and Quantify Brook Trout Populations in Headwater Streams of the Adirondack Mountains, New York, Transactions of the American Fisheries Society, 146:1, 99-111, DOI: 10.1080/00028487.2016.1243578

To link to this article: http://dx.doi.org/10.1080/00028487.2016.1243578

Published online: 06 Dec 2016.

Transactions of the American Fisheries Society 146:99–111, 2017

ISSN: 0002-8487 print / 1548-8659 online
DOI: 10.1080/00028487.2016.1243578

FEATURED PAPER

Efficacy of Environmental DNA to Detect and Quantify Brook Trout Populations in Headwater Streams of the Adirondack Mountains, New York

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Abstract

Environmental DNA (eDNA) analysis is rapidly evolving as a tool for monitoring the distributions of aquatic species. Detection of species' populations in streams may be challenging because the persistence time for intact DNA fragments is unknown and because eDNA is diluted and dispersed by dynamic hydrological processes. During 2015, the DNA of Brook Trout *Salvelinus fontinalis* was analyzed from water samples collected at 40 streams across the Adirondack region of upstate New York, where Brook Trout populations were recently quantified. Study objectives were to evaluate different sampling methods and the ability of eDNA to accurately predict the presence and abundance of resident Brook Trout populations. Results from three-pass electrofishing surveys indicated that Brook Trout were absent from 10 sites and were present in low (<100 fish/0.1 ha), moderate (100–300 fish/0.1 ha), and high (>300 fish/0.1 ha) densities at 9, 11, and 10 sites, respectively. The eDNA results correctly predicted the presence and confirmed the absence of Brook Trout at 85.0–92.5% of the study sites; eDNA also explained 44% of the variability in Brook Trout population density and 24% of the variability in biomass. These findings indicate that eDNA surveys will enable researchers to effectively characterize the presence and abundance of Brook Trout and other species' populations in headwater streams across the Adirondack region and elsewhere.

Biodiversity information is becoming increasingly important because species losses at local, regional, and global scales are increasing rapidly, are often difficult to detect or characterize, and can significantly impact the health of aquatic and terrestrial ecosystems (Valentini et al. 2016). Over the past three decades, an alternative tool has emerged for detecting the presence of organisms from aquatic and terrestrial environments by using species-specific DNA (Taberlet et al. 2012). Deoxyribonucleic acid is the hereditary material in organisms that contains the biological instructions for building and maintaining them. The chemical structure of DNA is the same for all organisms, but different nucleic acid sequences provide a means by which to

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Received April 15, 2016; accepted September 26, 2016

identify individual species. Thus, nuclear or mitochondrial DNA that is shed from an organism into its environment (environmental DNA [eDNA]) theoretically can be used to detect the presence or confirm the absence (hereafter, "presence/absence") of local species—and possibly can be used to quantify population sizes—in aquatic or terrestrial systems. Sources of eDNA include feces, mucus, gametes, shed skin, and carcasses. In aquatic environments, eDNA is diluted and distributed by currents and other hydrological processes. Intact sequences of DNA may be sequestered in water and/or sediments of aquatic systems for hours, days, years, or millennia depending on environmental conditions (Epp et al. 2010; Dejean et al. 2011; Thomsen and Willerslev 2015).

Analysis of eDNA is a new and rapidly evolving molecular genetics tool with great potential to facilitate the effective monitoring, management, and conservation of important fish resources. Environmental DNA can be detected via routine molecular techniques, such as PCR to amplify species-specific DNA sequences; thus, the presence of species can be detected without actually observing them. Species detection with eDNA may sometimes be difficult when target species are present at low densities, but detection can be especially problematic in aquatic environments due to the dynamic nature of various physical, chemical, and biological interactions. For example, the persistence and detectability of eDNA generally decrease with time after removal of the DNA source (Dejean et al. 2011) and can also vary with discharge, abundance of the target species, distance between the target species and the eDNA sampling point, and eDNA production rates (Jane et al. 2015; Thomsen and Willerslev 2015; Wilcox et al. 2016). Environmental DNA analysis is currently being evaluated for uses such as surveillance of invasive aquatic species, identification and monitoring of endangered species, and analysis of biodiversity (Thomsen and Willerslev 2015). These methods could enhance species detection and improve biodiversity assessments, especially for species that are rare or difficult to sample (Thomsen et al. 2012; Spear et al. 2015). Depending on information requirements, eDNA sample collection and analysis may also be more cost effective than traditional fish survey methods. Because all aquatic organisms naturally shed DNA into the water they occupy, eDNA can be analyzed to monitor the presence/absence and possibly the relative abundance of individual species as well as the richness of species assemblages in aquatic ecosystems (Mahon et al. 2013; Pilliod et al. 2013).

Due to a wide variety of stressors, the distribution of Brook Trout *Salvelinus fontinalis* and the diversity of native fish species have decreased steadily in streams and lakes across the northeastern USA over the past 100 years (Hudy et al. 2008); however, the losses of Brook Trout and other fish populations have been particularly precipitous in poorly buffered and remote areas of the western Adirondack Mountains due to acidic deposition (Schofield 1976; Baker and Christensen 1991). Over the past 10–15 years, implementation of the Clean Air Act of 1990 and related rules has led to decreases in sulfur and nitrogen oxide emissions, acidic deposition, and surface water toxicity (Lawrence et al. 2011; Waller et al. 2012; Driscoll et al. 2016); these changes may have improved water quality to the point that Brook Trout and other acid-tolerant (and acid-intolerant) native species are now able to recolonize formerly acidified streams and lakes. Increases in dissolved organic carbon levels in humic surface waters should also impede formation of labile or toxic inorganic fractions (species) of aluminum (Al_i), thus further improving water quality (Lawrence et al. 2013; Fakhraei and Driscoll 2015). However, no systematic state or federal program exists to monitor and assess the recovery of fish populations in streams and rivers of the Adirondack Mountains, partly because few tools are available to economically and accurately determine the presence/absence of Brook Trout and other fish species in the numerous streams and lakes occurring across such a large region.

In 2015, the U.S. Geological Survey (USGS) and the Adirondack Watershed Institute at Paul Smith's College initiated an investigation of eDNA sampling methods and their ability to correctly characterize the presence/absence of Brook Trout populations in headwater streams of the western Adirondack region in upstate New York. The primary objective of this study was to evaluate the efficacy of eDNA analysis as a rapid and cost-effective tool for assessing the status of Brook Trout populations (and entire fish communities) in headwater streams. Related goals of the study were to test and refine sampling methods, determine the accuracy of presence/absence predictions, and explore the ability of eDNA results to predict the density and biomass of Brook Trout populations.

METHODS

Quantitative fish community surveys were completed at study reaches in 40 streams located in or near the western Adirondack Mountains (Figure 1; Table 1) during summer 2014 and 2015. Sediment and water samples for eDNA analysis were collected at each of the study sites once from August 25 to September 2, 2015, and during revisits to three sites on September 21, 2015. Water (filters) and sediment samples were collected from each fish survey site, frozen on dry ice, and transported to Paul Smith's College for analysis, as described in detail below.

Fish surveys.—The composition of fish communities, including the density and biomass of Brook Trout populations, in all stream reaches were characterized during quantitative surveys performed in summer 2014 and 2015 as part of a separate study (B. Baldigo; https://www.sciencebase.gov/catalog/item/55ccf335e4b08400b1fe1177), generally following the methods described by Baldigo et al. (2008). In brief, fish assemblages were quantified in seine-blocked reaches that ranged in length from 10 to 20 mean channel widths and often encompassed one or two complete geomorphic channel unit sequences (Simonson et al. 1994; Fitzpatrick et al. 1998; Meador et al. 2003). In each study reach, all observed fish

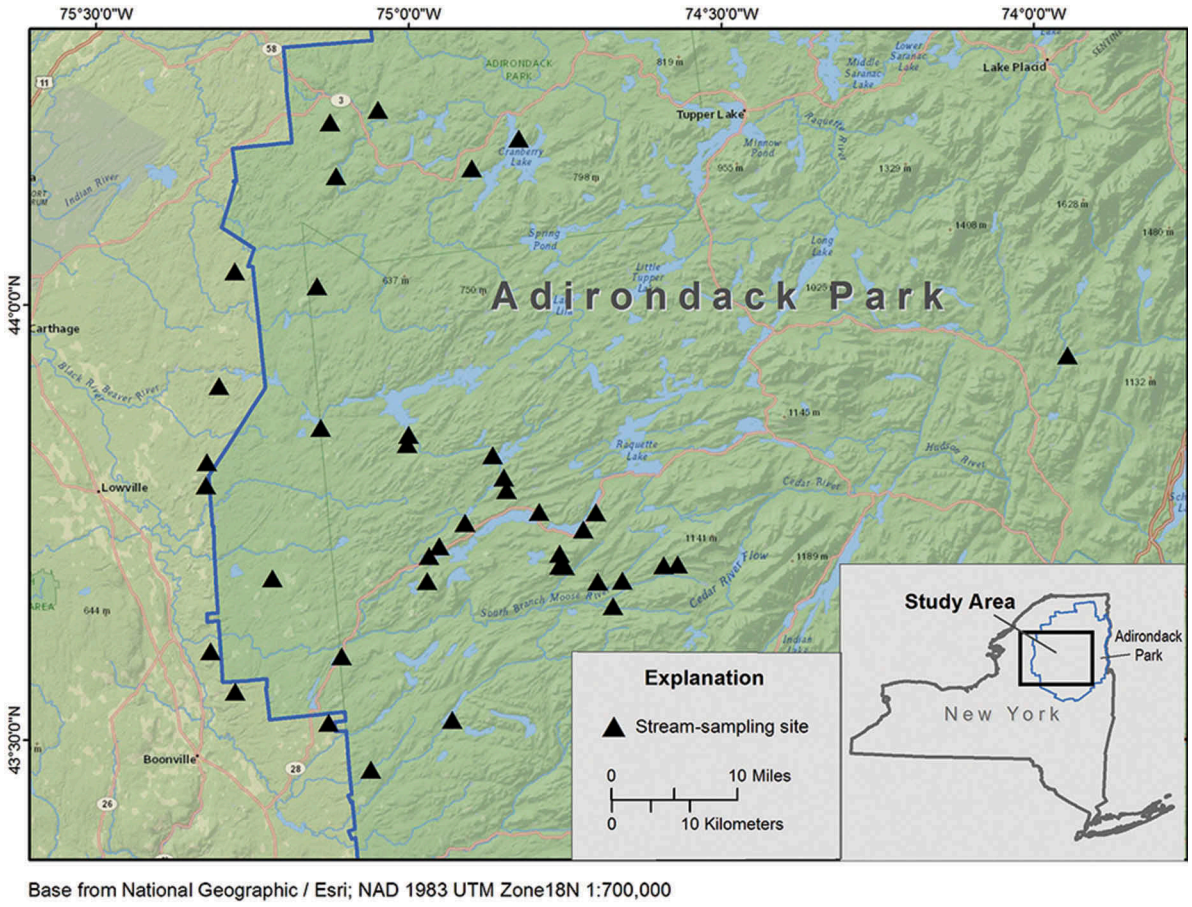

FIGURE 1. Locations of 40 stream sites, primarily in the western Adirondack Mountains, where fish community surveys were completed during summer 2014 and 2015 and where Brook Trout eDNA samples were collected during August and September 2015.

were stunned by using a backpack electroshocker and were collected by two or three netters during each of three passes. The total length and mean width of each study reach were measured on site and were used to calculate the total area sampled. Fish that were collected during each pass were identified to species; the lengths and weights for all rare and abundant species (for individuals >150 mm) were recorded. The lengths and weights for some small and abundant species (e.g., some minnows) were obtained from 30 individuals, after which pooled weights (and counts) were recorded for each species in batches of 2–20 fish. All fish were returned to the stream after they were processed, and all passes were finished. The total number and weight or biomass of Brook Trout (and 95% confidence intervals [CIs]) were estimated for each site by using a maximum likelihood population estimator built on inherent assumptions in the Moran–Zippin method of proportional reduction (Zippin 1958; Van Deventer and Platts 1985). The two values were divided by the total area sampled to estimate the density (number of fish per 0.1 ha) and biomass (total g of fish per 0.1 ha) of the Brook Trout population at each study site. The resulting density and biomass values were not excessively large (as if expressed in units of m^2) or small (as if expressed in units of km^2) and were comparable to the actual number and mass of fish that might be observed in a representative 10-m-wide × 100-m-long (1,000-m^2) sampling reach.

Sediment and water sampling for eDNA.—At each study site, one unused 500-mL Nalgene bottle, forceps, a filter holder (stand and funnel), and a spatula (for sediments) were decontaminated for 5 min in a solution of 10% Clorox and 90% site water and were rinsed 10 times in water from the site. One water sample and one sediment sample were collected at each site during the first visit (August 25–September 2, 2015); however, duplicate water samples were collected from three sites that were resampled on September 21, 2015, to investigate alternative sampling methods (field replicates) and potential contamination issues. For each eDNA water sample, up to 6 L of stream water were collected with the unused 500-mL bottle, poured (up to 12 bottles; one at a time) into the filter holder, and vacuumed through a 47-mm-diameter, glass-fiber filter (1.5-µm pore size; Whatman, Pittsburgh, Pennsylvania) on site by using a hand pump. The volume of water that was successfully

TABLE 1. Site identification code (ID), stream name, coordinates (North American Datum of 1983 [NAD83]), U.S. Geological Survey (USGS) station number, drainage area, elevation, and sampling reach length and width for 40 Adirondack streams where Brook Trout populations and Brook Trout DNA were characterized during 2014 and 2015.

Site ID	Stream name	Latitude, ºN (NAD83)	Longitude, ºW (NAD83)	USGS station number	Drainage area (km^2)	Elevation (m)	Mean site width (m)	Total site length (m)
6020	Yellow Creek	44°12'42.9″	75°07'31.4″	441242075073401	2.54	387	2.5	49
7003	Unnamed	44°09'00.3″	75°06'56.9″	440859075065901	2.20	414	2.3	51
7028	Unnamed	44°13'35.0″	75°02'55.3″	441332075024401	0.85	387	1.8	52
8011	Unnamed	44°09'32.3″	74°53'57.7″	440931074540101	0.38	482	1.3	46
11010	Browns Creek	44°02'26.3″	75°16'35.8″	440239075165601	2.94	325	3.6	50
17009	Unnamed	43°54'31.6″	75°18'03.4″	435431075180201	1.42	329	1.0	53
21005	South Branch Crystal Creek	43°47'39.7″	75°19'16.1″	434739075191601	5.52	337	0.8	45
22004	Unnamed	43°50'31.9″	75°00'08.8″	435032075000901	0.72	557	1.0	48
22017	Unnamed	43°51'39.8″	75°08'21.8″	435139075082201	2.30	460	2.1	55
26031	Fish Creek	43°41'16.3″	75°12'54.8″	434116075125501	8.62	413	4.1	65
28006	Benedict Creek	43°41'01.5″	74°42'04.2″	434101074420301	15.30	576	4.7	81
28018	Unnamed	43°41'04.7″	74°39'42.2″	434105074393501	1.03	570	1.0	30
28022	Bradley Brook	43°42'08.5″	74°35'45.9″	434208074354501	9.57	643	3.4	76
28024	Silver Run	43°42'11.6″	74°34'26.9″	434208074343001	4.70	699	3.6	67
28037	Unnamed	43°39'20.5″	74°40'35.4″	433920074403401	1.21	582	1.3	45
29008	Beauty Creek	43°36'13.2″	75°18'45.9″	433613075184301	0.46	317	1.9	55
29012	Unnamed	43°33'26.3″	75°16'26.8″	433324075165001	0.77	348	1.0	47
30003	Caroline Creek	43°31'18.5″	75°07'33.9″	433117075073501	2.27	426	3.1	66
30009	Unnamed	43°35'53.8″	75°06'20.3″	433553075062101	0.85	479	2.0	50
31007	Unnamed	43°31'30.3″	74°55'51.7″	433130074555201	1.91	579	2.5	50
35008	Unnamed	43°28'05.9″	75°03'34.1″	432806075033501	1.06	451	1.2	56
21013-D	Unnamed	43°49'15.9″	75°19'11.5″	434915075190901	3.66	325	2.7	68
Birch	Birch Creek	43°51'11.2″	75°00'00.0″	435111075000001	7.40	526	3.5	54
Black	Black Bear Brook	43°45'50.7″	74°47'35.4″	434550074473501	2.71	534	2.3	57
Bald	Bald Mountain Brook	43°45'05.6″	74°54'40.7″	04253770	1.86	544	2.2	62
Buck-D	Buck Creek	43°44'37.9″	74°43'23.0″	04253296	3.04	547	2.9	102
Durgin-D	Durgin Brook	43°56'21.2″	73°57'10.3″	01315170	17.47	529	6.1	110
Fly-D	Fly Pond Outlet	43°45'05.3″	74°54'35.9″	04253775	0.83	544	1.7	50
Moss-D	Moss Lake Inlet	43°47'21.2″	74°50'40.8″	04253715	2.79	539	1.5	57
Pancake	Pancake Hall Creek	43°49'45.6″	74°51'59.1″	434945074515901	0.83	570	1.7	55
Seventh-D	Seventh Lake Inlet	43°45'47.8″	74°42'11.7″	04253291	6.71	552	4.3	86
Wheeler	Wheeler Creek	43°42'48.3″	74°58'03.0″	434417074445401	6.50	551	3.8	57
Windfall	Windfall Pond Outlet	43°48'13.8″	74°50'57.6″	434813074505701	4.26	540	1.9	75
Beaver	Beaver Brook	43°43'29.6″	74°57'06.7″	434329074570601	1.06	546	0.9	60
Nicks	Nicks Creek	43°41'05.2″	74°58'15.4″	434105074581501	2.99	585	2.6	58
9008	Unnamed	44°11'34.6″	74°49'27.2″	441137074492001	0.72	456	2.1	50
12008	Unnamed	44°01'25.0″	75°08'43.8″	440125075084201	0.65	461	1.3	52
27019	Unnamed	43°42'57.2″	74°45'40.9″	434256074453801	1.32	600	1.5	54
27022	Unnamed	43°42'07.5″	74°45'39.1″	434207074453801	1.37	612	0.7	60
27025	Unnamed	43°42'06.6″	74°45'11.0″	434208074450901	0.62	612	0.5	100

filtered (to refusal) for each sample was recorded, as were the site identification code (ID), date, and time of sampling. The filter was removed from the stand with sterile forceps, rolled up (dirty side in), placed into a labeled vial and an unused plastic bag, and immediately placed on dry ice. For each eDNA sediment sample, several grams of silty sediment were collected from the streambed in slackwater areas by using a spatula and were placed into an unused plastic bag; the sample was double-bagged and labeled with the site ID, date, and time and then was placed onto dry ice. All samples were kept on dry ice and were delivered to Paul Smith's College within 4–48 h of the time of collection.

Environmental DNA analysis.—Environmental DNA was extracted and purified from the glass-fiber filters and from the sediment samples (silt and clay fractions) by using the PowerWater DNA Isolation Kit and the PowerSoil DNA Isolation Kit, respectively (MoBio Laboratories, Inc., Carlsbad, California), in accordance with the manufacturer's protocols. Silt and clay fractions were prepared by first mixing sediments with five volumes of sterile, type II water and allowing sand particles to settle for 1 min; the supernatant (containing silt and clay) was then centrifuged at 10,000 × *g* for 2 min to produce a pellet, 250 mg of which were used in the purification. Detection of Brook Trout DNA was conducted using the TaqMan Minor Groove Binder assay (described by Wilcox et al. 2013) and targets Brook Trout mitochondrial cytochrome *b*. Quantitative PCR was carried out by using a Step One Real-Time PCR System (Thermo Fisher Scientific, Inc., Waltham, Massachusetts) that was housed in a dedicated facility. The following cycling protocol was used: an initial hold for 10 min at 95°C; 40 cycles of 15 s at 95°C (denaturation); and 1 min at 60°C (annealing and extension). All reactions used the Environmental Master Mix 2.0 (Thermo Fisher Scientific) in a 20-μL final volume. Reaction components were as follows: 250-nmol Brick gene brk2 probe; 900-nmol brk2 primers; and VIC-dye-labeled internal positive control (IPC) primer/probe and IPC target DNA (Thermo Fisher Scientific), where the forward primer was CCACAGTGCTTCACCTTCTA TTTCTA, the reverse primer was GCCAAGTAATATAG CTACAAAACCTAATAGATC, and the FAM-dye-labeled probe was ACTCCGACGCTGACAA. One microliter of eluted DNA was used in each assay, and samples were run in triplicate. Average cycling threshold (C_t) values were calculated from the laboratory triplicates (pseudo-triplicates) that were analyzed from each sample. In samples with low DNA concentrations, not all triplicates produced C_t values (<40 cycles). In these cases, average C_t was calculated by using only the positive values. A no-template control was included with each assay to evaluate potential nucleic acid contamination and nonspecific amplification products that might yield false positives.

A standard curve was constructed over five orders of magnitude to determine the relative concentrations of Brook Trout DNA in environmental samples by using total genomic DNA purified from local Brook Trout tissues (2 pg to 20 ng added to a 20-μL PCR). When the logarithm of starting material (ng of total genomic Brook Trout DNA) (*x*-axis) was plotted against the average C_t value (*y*-axis), the resulting line had a slope of −3.615, a *y*-intercept of 26.685, and an R^2 value of 0.998. The PCR efficiency was calculated as $[10^{(-1/\text{slope})}] - 1$, yielding an efficiency value of 89.1%. Based on this standard curve, the relative concentration of Brook Trout DNA in all environmental samples was calculated as

$$\text{Concentration } (\text{ng}/\mu\text{L}) = 10^{[(C_t - 26.685)/-3.615]}.$$

Hereafter, the relative concentration of Brook Trout DNA in environmental samples will be referred to as Brook Trout DNA or simply as eDNA. Because DNA is affected by so many factors, the lack of detectable DNA in a sample does not imply 100% confidence that no individuals were present. Thus, the phrase "a low probability of occurrence" may more closely represent the meaning of the term "absence" used in our analysis, results, and interpretations.

Data analysis.—Water and sediment samples were obtained from 40 fish survey sites where Brook Trout were known to be absent or where Brook Trout populations were identified as present in low to high densities based on prior fish surveys. Environmental DNA was assessed in water samples from all 40 sites and in sediment samples from only 10 sites. The efficacy of eDNA analyses for characterizing the presence/absence of Brook Trout populations was determined by evaluating the proportion of sites where eDNA correctly classified Brook Trout populations as being present or absent. On September 21, 2015, duplicate water samples were collected at three sites that were initially misclassified by eDNA results; this was done to address the possibility of cross-contamination, inadequate decontamination procedures, and the use of field replicates (for increased sample volume). Except for analysis of presence/absence results (which were evaluated by using initial and final eDNA data sets), all analyses utilized the revised eDNA data from the three resampled sites. The relationships between Brook Trout population density or biomass and the eDNA at all 40 sites were defined by using linear regression analysis of both raw data and log-transformed data. The relationships were considered significant at *P*-values less than or equal to 0.05. Resultant equations may be used to predict the relative or quantified abundance (density) and biomass of Brook Trout populations in headwater streams based on eDNA results.

RESULTS

Brook Trout Populations

Brook Trout were absent from 10 sites and were estimated (using single-survey data from 34 sites and mean data from the 6 sites where duplicate surveys were completed) to be present in low densities (<100 fish/0.1 ha) at 9 sites, moderate densities (100–300 fish/0.1 ha) at 11 sites, and high densities

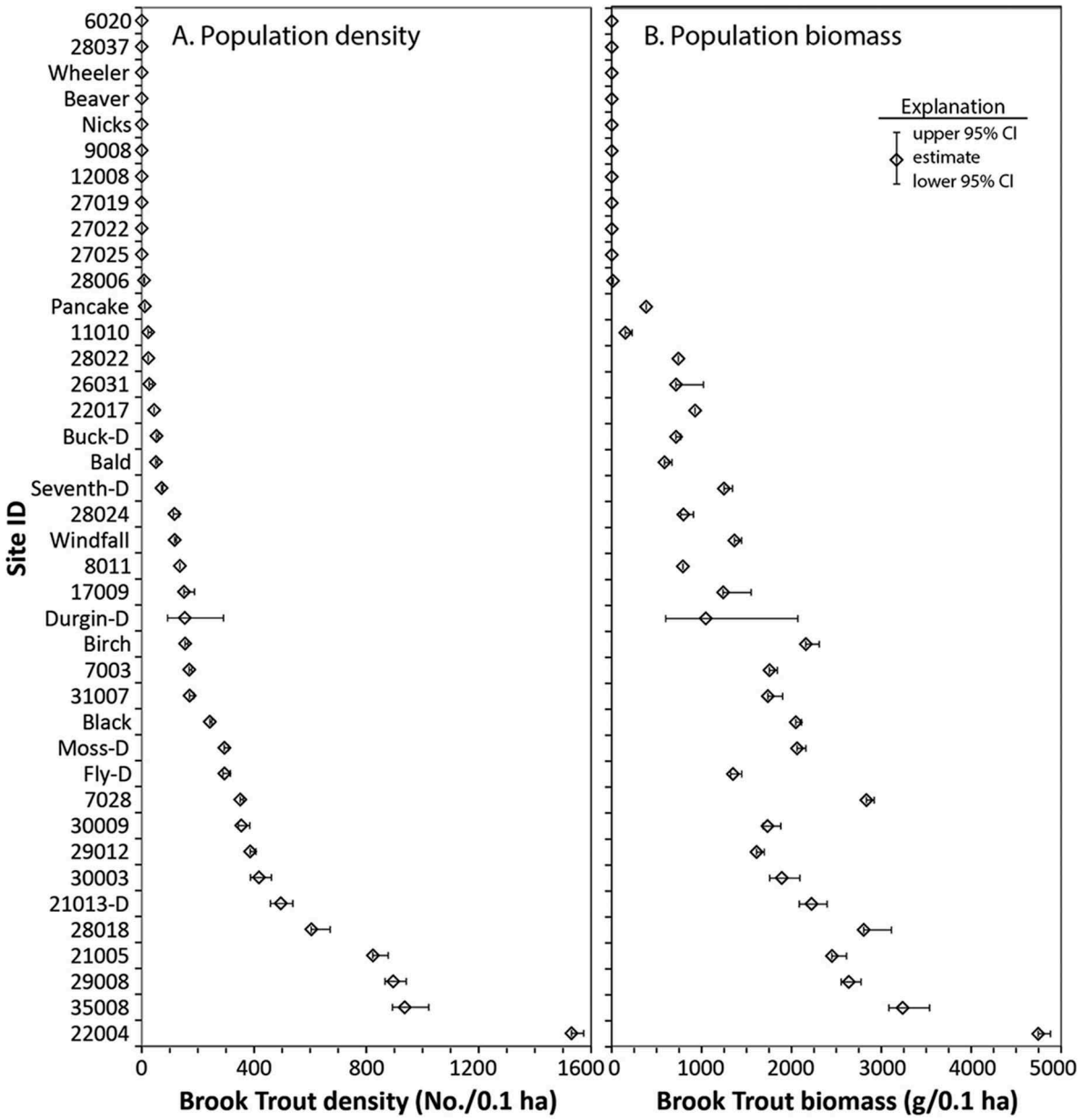

FIGURE 2. Estimates (±95% confidence interval) of Brook Trout **(A)** population density and **(B)** population biomass from 40 streams surveyed in the Adirondack region during summer 2014 and 2015 (site identification codes [ID] are defined in Table 1).

(>300 fish/0.1 ha) at 10 sites (Figure 2A; Table 2). Estimated Brook Trout biomass was zero at the 10 sites where they were not collected; biomass estimates ranged from 12.4 to 4,746 g/0.1 ha at the 30 sites where populations (at least 1 individual) were evident (Figure 2B; Table 2). Brook Trout biomass was low (12.4–1,000 g/0.1 ha) at 10 sites, intermediate (1,000–2,000 g/0.1 ha) at 10 sites, and high (>2,000 g/0.1 ha) at 10 sites (Figure 2B; Table 2). The species names, lengths, and weights of all fish that were collected during successive electrofishing passes at each of the 40 study sites are available online (Baldigo and George 2016).

Environmental DNA

A C_t value less than 40 in at least one of the laboratory triplicates was used as an indication of Brook Trout DNA presence in water or sediment samples. The failure to amplify the target in all triplicates indicated that concentrations in the eDNA sample were either nonexistent or below the lower limits of assay quantitation. Average C_t values for positive water samples ranged from 30.01 to 38.73, which spanned a 240-fold range in relative concentrations of Brook Trout DNA from 0.1200 to 0.0005 ng/µL (Table 2). The C_t values for the first 10 sediment samples (chosen at random) were all greater

TABLE 2. Summary of fish survey sampling area for each site (site identification codes [ID] are defined in Table 1); estimates of Brook Trout population density and biomass; and volumes of water filtered, collection dates, detection of Brook Trout DNA (environmental DNA [eDNA]), number of positive replicates, mean cycling threshold for water and sediment, and relative concentration of Brook Trout DNA in water from 40 Adirondack streams investigated during 2014 and 2015 (na = not applicable).

		Brook Trout population		Brook Trout eDNA samples					
Site ID	Fish survey area (m^2)	Density (fish/ 0.1 ha)	Biomass (g/ 0.1 ha)	Collection date (2015)	Volume of water filtered (mL)	Number of positive water replicates	Mean cycling threshold in water	Relative DNA concentration in water (ng/μL)	Mean cycling threshold in sediment
6020[a]	124.0	0.0	0.0	Aug 27	2,250	1	35.37	0.003958	na
				Sep 21	2,250	0	>40.0	na	na
28037	60.1	0.0	0.0	Aug 29	2,325	0	>40.0	na	na
Wheeler	218.9	0.0	0.0	Aug 26	275	0	>40.0	na	na
Beaver	52.4	0.0	0.0	Aug 26	3,000	0	>40.0	na	na
Nicks	148.2	0.0	0.0	Aug 26	375	0	>40.0	na	na
9008	106.0	0.0	0.0	Aug 27	6,000	0	>40.0	na	>40.0
12008	68.9	0.0	0.0	Sep 2	400	0	>40.0	na	na
27019	81.5	0.0	0.0	Aug 29	6,000	0	>40.0	na	na
27022	258.4	0.0	0.0	Aug 26	400	0	>40.0	na	na
27025	<20.0	0.0	0.0	Aug 29	1,100	0	>40.0	na	na
28006[b]	379.6	7.9	12.4	Aug 29	470	0	>40.0	na	na
Pancake[b]	93.5	10.7	382.9	Aug 26	1,750	0	>40.0	na	na
11010	177.5	22.5	151.0	Sep 2	900	1	36.38	0.002080	na
28022[a]	258.4	23.2	741.5	Aug 29	2,275	0	>40.0	na	na
				Sep 21	7,500	6	36.30	0.002189	na
26031[b]	263.2	26.6	714.4	Sep 2	2,000	0	>40.0	na	na
22017	113.6	44.0	927.1	Aug 29	2,500	2	36.49	0.001940	na
Buck-D	326.5	50.3	714.4	Aug 26	5,250	1	34.71	0.006027	na
Bald	134.5	52.0	586.4	Aug 26	2,000	1	35.96	0.002718	na
Seventh-D	382.9	70.5	1,248.7	Aug 26	575	1	38.73	0.000466	na
28024	239.9	116.7	797.1	Aug 29	6,000	3	31.13	0.058814	na
Windfall	145.1	117.1	1,364.3	Aug 26	4,500	3	36.98	0.001417	na
8011	58.9	135.9	791.4	Aug 27	6,000	2	37.31	0.001154	>40.0
17009	53.3	150.2	1,239.1	Sep 2	4,000	3	33.53	0.012779	na
Durgin-D	614.7	153.1	1,045.1	Aug 27	6,000	3	32.83	0.020001	na
Birch	187.6	154.5	2,159.3	Aug 29	1,250	3	34.36	0.007548	>40.0
7003	118.3	169.0	1,754.6	Aug 27	4,750	3	32.97	0.018295	na
31007	123.5	170.0	1,736.8	Aug 25	6,000	2	36.18	0.002370	na
Black[a]	132.1	242.2	2,048.0	Aug 26	6,000	0	>40.0	na	na
				Sep 21	12,000	6	32.10	0.031774	na
Moss-D	113.4	293.8	2,062.3	Aug 26	1,650	3	34.77	0.005788	>40.0
Fly-D	90.4	294.2	1,348.0	Aug 26	5,500	3	31.30	0.052778	>40.0
7028	94.1	350.6	2,833.6	Aug 27	4,375	3	31.64	0.042501	>40.0
30009	98.8	354.4	1,733.7	Aug 30	6,000	3	30.09	0.114312	>40.0
29012	49.2	386.1	1,611.5	Sep 2	6,000	3	31.83	0.037737	>40.0
30003	201.3	417.3	1,891.7	Sep 2	6,000	3	30.01	0.120033	na
21013-D	204.3	494.6	2,222.3	Sep 2	6,000	3	33.15	0.016244	na
28018	29.8	603.6	2,803.5	Aug 29	6,000	3	31.52	0.045975	na
21005	36.4	823.0	2,449.9	Sep 2	4,500	3	33.69	0.011516	>40.0
29008	105.1	894.8	2,635.9	Sep 2	6,000	3	32.75	0.021047	na
35008	69.4	936.1	3,235.9	Aug 30	6,000	3	33.12	0.016558	>40.0
22004	45.7	1,530.3	4,746.0	Aug 29	2,750	3	31.55	0.045104	na

[a]Sites with false-positive or false-negative eDNA results that were modified after being resampled.

[b]Sites that were initially misclassified (false-positive or false-negative eDNA results) but were not resampled.

than 40, which indicated the absence of detectable Brook Trout DNA in stream sediments. Brook Trout populations were absent at one of these sites (site 9008) but ranged from 90 to 936 fish/0.1 ha at the nine other sites where eDNA was not detected in sediments (Table 2). Consequently, eDNA was not analyzed from sediment samples collected at the remaining 30 sites. The negative results in all sediment samples could not have been caused by PCR inhibition because the IPCs amplified efficiently in all analyzed sediment samples.

Relationship of eDNA to Brook Trout populations

The eDNA results indicated that water was more effective than sediment at retaining the genetic material exuded by Brook Trout in the surveyed headwater streams. As described above, Brook Trout DNA was not detected in any of the 10 sediment samples that were analyzed, which included nine sites where Brook Trout populations were observed (Table 2). Sediment eDNA samples, therefore, were 0% correct in classifying sites where Brook Trout were present. The lack of positive detections meant that relationships between the relative eDNA concentration in sediments and the Brook Trout population metrics could not be explored.

The eDNA data from the first set of water samples (August 25–September 2, 2015) correctly detected the presence of Brook Trout at 25 of the 30 sites (83% correct classification) where Brook Trout populations were observed during fish surveys in 2014 or 2015 (Table 2). The eDNA data from the initial survey and from additional samples collected on September 21, 2015 (at 2 of the 5 sites where individuals were collected but where eDNA was not detected), correctly detected the presence of Brook Trout at 27 of the 30 sites (90% correct classification) where their populations were evident (Table 2). The remaining three sites that were incorrectly classified by eDNA as containing no Brook Trout were not resampled. Environmental DNA results correctly confirmed the absence of Brook Trout at 9 of 10 sites (90% correctly classified) where they were not observed during the first set of eDNA samples and at all 10 sites (100% correctly classified) after resampling (September 21, 2015) of the one site that was misclassified as having Brook Trout present (Table 2). The eDNA in water samples correctly predicted the presence or confirmed the absence of Brook Trout populations at 85% of sites after the first set of samples and at 92.5% of sites after duplicate water samples were collected and analyzed from three misclassified sites. However, the resampling of several sites to correct for known presence or absence artificially increased the apparent accuracy of our classifications.

The direct relationship between Brook Trout classifications from eDNA and population density was generally comparable to the relationship between classifications from eDNA and population biomass (Figure 3). The relative concentration of Brook Trout DNA was able to explain a moderate amount of variability (44%) in the density of local Brook Trout populations (Figure 3A) but only 24% of the variability in population biomass (Figure 3B). These differences indicated that the amount of genetic material that was suspended in the water column corresponded more closely to the number of resident individuals than to the total mass of resident individuals within any given stream reach. The resulting models (equations), 95% CIs, and 95% prediction intervals indicated that eDNA could be used to predict the abundance (with known levels of error) of Brook Trout populations in these and other streams within the Adirondack region.

DISCUSSION

The present study not only confirmed the ability of eDNA to accurately predict the presence/absence, relative abundance, and biomass of wild Brook Trout populations in streams but is one of the first studies to investigate the ability of eDNA to quantify the density and biomass of Brook Trout populations across a large geographic region. Environmental DNA correctly classified the presence/absence of Brook Trout in 85.0–92.5% of the 40 streams where fish populations were surveyed. Though speculative, the collection of additional eDNA field replicates (more volume) at the three other misclassified sites might have increased the accuracy of presence/absence predictions to nearly 100%. The relative concentrations of Brook Trout DNA were also able to explain 44% of the variability in density and 24% of the variability in biomass of Brook Trout populations. These findings were generally analogous to the results from several recent investigations of Brook Trout eDNA. For example, Wilcox et al. (2016) compared the results of eDNA and electrofishing surveys at 46 sites in 16 first-order Montana streams and showed that eDNA could detect the presence of Brook Trout with over 99% accuracy when densities were greater than 1 fish per 100 m of stream length. Although Wilcox et al. (2016) used single-pass electrofishing at most sites and no block nets, eDNA copy number was strongly correlated ($R^2 = 0.59$) with the estimated density of Brook Trout that were longer than 75 mm. In addition, Jane et al. (2015) showed that eDNA from Brook Trout held in cages within two fishless headwater streams could be detected with 100% accuracy at least 240 m downstream from its source and that eDNA copy number was positively correlated with the total biomass of caged fish. In contrast, extremely low DNA concentrations were identified as the primary reason why eDNA was not endorsed as a tool for assessing the relative abundance of Brook Trout in High Lake, Oregon (Blankenship et al. 2011). Although the strong relationships noted in the present study and in the Wilcox et al. (2016) and Jane et al. (2015) studies indicate that the absolute density and biomass of wild Brook Trout populations can be predicted or inferred from eDNA, the relatively high levels of uncertainty (error) could limit the utility of this method.

The primary objective of our study was to determine whether eDNA samples could accurately quantify the density and biomass of Brook Trout populations in small streams. We

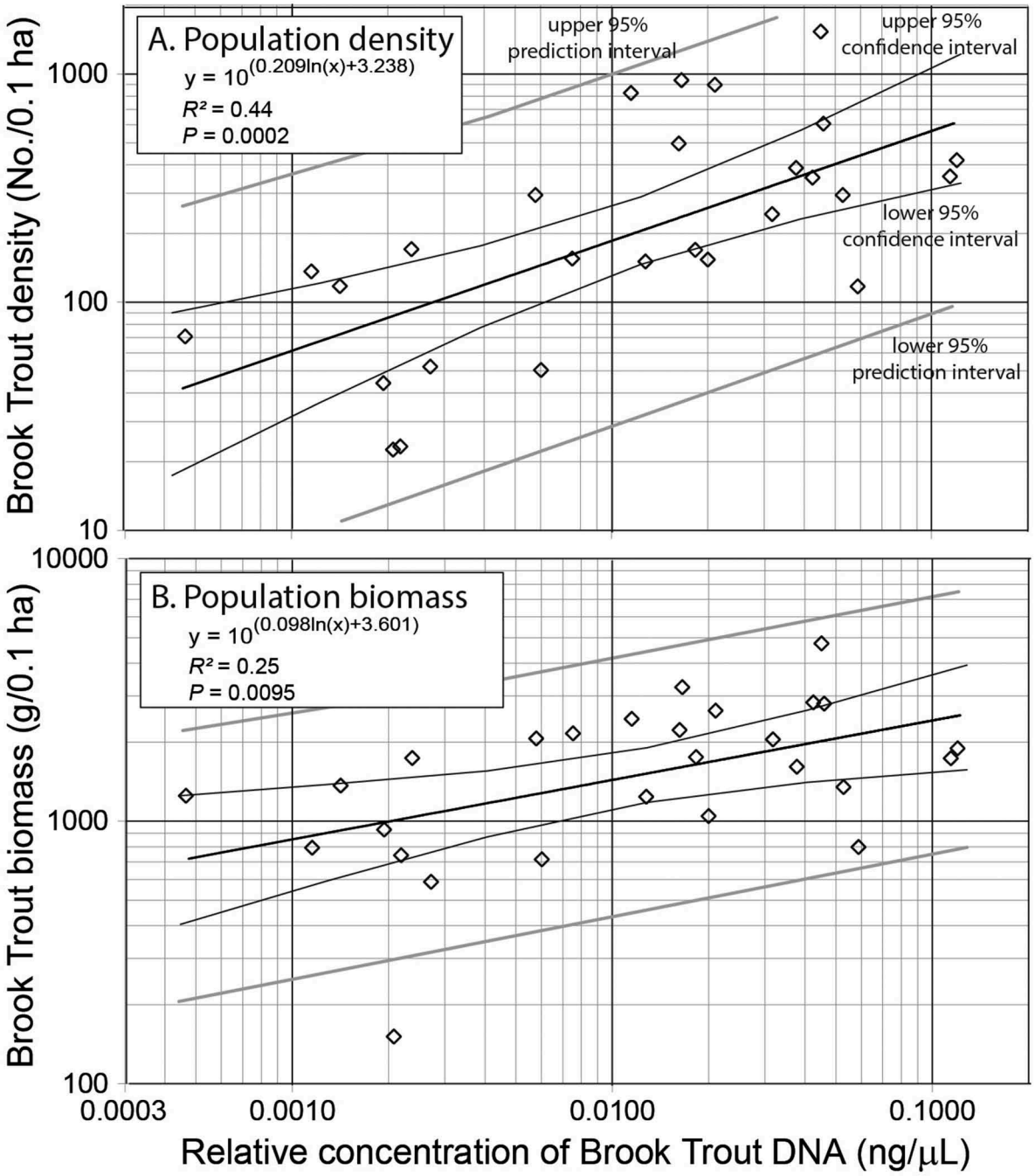

FIGURE 3. Relationships between the relative concentration of Brook Trout DNA and quantitative estimates of Brook Trout **(A)** population density and **(B)** population biomass in 40 streams of the Adirondack region, 2014–2015. The regression lines (bold black lines) are bounded by 95% confidence intervals (thin black lines) for both equations and by 95% prediction intervals (gray lines) for population density and biomass data.

achieved this objective as discussed above; however, the use of eDNA samples to predict the relative abundance of resident Brook Trout may be more easily supported. Although the 95% CIs of the predictive equations for Brook Trout density and biomass were relatively narrow, the 95% prediction intervals for observed density and biomass values were quite large (Figure 2). For example, the equation and prediction intervals for density (Figure 2A) indicated that Brook Trout populations at sites with a relative eDNA concentration of 0.001 ng/µL might average a density near 60 fish/0.1 ha, yet the observed densities varied from about 10 to 350 fish/0.1 ha. At a higher relative eDNA concentration of 0.1 ng/µL, about 550 fish/0.1 ha would be predicted, whereas most density observations ranged from 90 to 3,000 fish/0.1 ha. Likewise, biomass

predictions using the same two eDNA concentrations (0.001 and 0.1 ng/μL) averaged 850 and 2,500 g/0.1 ha, respectively, but the observed biomass ranged from 250 to 2,300 g/0.1 ha and from 750 to 7,000 g/0.1 ha, respectively (Figure 2B). Understandably, predicting the relative density or biomass of Brook Trout populations from eDNA concentrations would be less error prone than quantifying population density or biomass. In general, we found that (1) undetectable relative eDNA concentrations (0 ng/μL) denoted the absence of Brook Trout populations (no density or biomass); (2) eDNA concentrations from 0.0003 to 0.003 ng/μL indicated low density (<100 fish/0.1 ha) and low biomass (<1,000 g/0.1 ha); (3) eDNA concentrations between 0.003 and 0.03 ng/μL predicted moderate population density (100–300 fish/0.1 ha) and moderate biomass (1,000–2,000 g/0.1 ha); and (4) eDNA concentrations greater than 0.03 ng/μL predicted high population density (>300 fish/0.1 ha) and high biomass (>2,000 g/0.1 ha). Obviously, both relative and quantified fishery data are rarely needed to address specific questions concerning the condition (and/or distribution) of targeted species or entire fish assemblages in aquatic systems. Thus, the application of eDNA sampling, qualitative fish surveys, or quantitative fish surveys will depend on the specific objectives of any given investigation.

The ability of eDNA samples to detect the presence of Brook Trout or other aquatic species could vary widely in streams depending on discharge (dilution of eDNA), distance between the target species and the eDNA sampling point, the production and persistence of eDNA, species abundance, and several other factors. Production of eDNA from aquatic species is associated with normal sloughing of external (skin and gill) and internal (intestinal) tissues into surrounding waters (Thomsen and Willerslev 2015) and can vary with diet (Klymus et al. 2015) and reproductive stage (Maruyama et al. 2014). Production of eDNA may sometimes—but not always—be enhanced by increased temperature (Takahara et al. 2012), crowding (related to low summer flows), fish activity (related to high stormflows), and chemically stressful conditions (Maruyama et al. 2014; Pilliod et al. 2014; Klymus et al. 2015; Strickler et al. 2015; Wilcox et al. 2016). Increased discharge and distance from caged Brook Trout were shown to reduce the eDNA copy number in two fishless streams (Jane et al. 2015), possibly due to differences in DNA settling rates, dilution, and/or degradation (Turner et al. 2015). Jane et al. (2015) also postulated that low flows (and low velocities) could promote cell settling, whereas high flows could dilute eDNA. In the same study, Jane et al. (2015) noted that "the presence of inhibitors resulted in no amplification for high copy number samples in the absence of an inhibition-releasing strategy." High concentrations of organic substances (e.g., humic and fulvic acids) have in fact been reported to inhibit DNA amplification in environmental samples (Matheson et al. 2010; Sigsgaard et al. 2015). Although dissolved organic carbon concentrations ranged up to 470 μmol carbon/L in many of our tannin-stained streams (our unpublished data), inhibition was not evident (i.e., Brook Trout DNA was strongly amplified in all IPCs) due to the use of TaqMan Environmental Master Mix, which efficiently "releases" PCR inhibition (Takahara et al. 2015). Additionally, eDNA from amphibians and fish has reportedly reached undetectable levels within a few hours to a few days or weeks after removal of the target species from natural streams or experimental mesocosms (Dejean et al. 2011; Thomsen et al. 2012; Pilliod et al. 2014). Relative eDNA concentrations or the number of copies of the mitochondrially encoded cytochrome-*c* oxidase I gene complex have been observed to be correlated with the density and/or biomass of aquatic species (Takahara et al. 2012; Wilcox et al. 2016; present study). The Brook Trout populations in the present study were sampled several weeks prior to and/or 1 year prior to the eDNA sample collections (from the bottom of 45–110-m-long fish survey reaches), and repeated surveys in consecutive years at six of the sites confirmed that there were no measurable changes in the population metrics. These findings suggest that Brook Trout populations in our study reaches were stable between years and were representative of densities and biomass across larger stream segments. Thus, it is unlikely that our eDNA results were affected by population swings, the persistence of sloughed DNA, or the distance between eDNA sampling points and resident Brook Trout. Based on the occurrence of several false negatives, however, clearly there is some minimum population density level below which Brook Trout DNA production, dilution, and PCR quantitation act to limit the detection of eDNA in Adirondack streams.

Results from Wilcox et al. (2016) and the present study provide evidence for a minimum Brook Trout density (threshold) above which eDNA can be used to effectively detect wild populations in small streams. Wilcox et al. (2016) reported a probability of 0.99 for detecting Brook Trout in reaches of first-order stream with at least 3 fish/100 m (or 1 fish/100 m^2 given that their study reaches averaged 3 m wide) when 5–10 samples were analyzed. Those authors also noted that the detection probability was much lower (0.18) at a Brook Trout density of 1 fish per 1,000 m of stream (0.3 fish/100 m^2). In the Adirondack Mountains, Brook Trout eDNA was detected in two streams (1 site was sampled twice) with densities of roughly 22.5 and 23.2 fish/0.1 ha (2 fish/100 m^2), but detection was unsuccessful in three streams (none sampled twice) with densities of 7.9, 10.7, and 26.6 fish/0.1 ha (0.8–2.7 fish/100 m^2; Table 2). Our results and those of Wilcox et al. (2016) indicate that Brook Trout DNA is generally above detectable thresholds in streams where population densities are greater than 10–20 fish/0.1 ha (1–2 fish/100 m^2) when multiple samples (field replicates) are analyzed. Although low levels of eDNA may have limited our ability to detect Brook Trout populations in several small Adirondack streams, relative concentrations of eDNA greater than 0.0003 ng/μL generally indicated the presence of at least 2 Brook Trout/100 m^2.

The absence of detectable Brook Trout DNA in all sediment samples from headwater streams with large Brook Trout populations was unexpected because aquatic sediments have been demonstrated as important sources of fish eDNA in aquatic systems, sometimes persisting for thousands of years (Thomsen and Willerslev 2015). For example, Stager et al. (2015) detected the eDNA of Yellow Perch *Perca flavescens* in sediment cores from strata that were dated 2,200 years old in an Adirondack lake. More importantly, Turner et al. (2015) reported that concentrations of fish eDNA were consistently higher (albeit more variable) in samples from bed sediments than from surface waters in both lakes and rivers. Though inhibitory contaminants can negate PCR results (Pilliod et al. 2014; Jane et al. 2015), PCR inhibition was not the cause of nondetection in our stream sediment samples, as indicated by the strong amplification of Brook Trout DNA in all IPCs. Several factors can also affect the degradation or persistence of eDNA under natural conditions. Although the mechanisms may not be well understood, eDNA persistence generally increases under anoxic conditions (Epp et al. 2010; Dejean et al. 2011; Rees et al. 2014; Thomsen and Willerslev 2015). The absence of detectable Brook Trout DNA in stream sediments might be explained by rapid movement and clearing of sediments or enhanced DNA degradation due to high oxygen concentrations (or ultraviolet exposure), which are seldom pertinent in deep lakes and rivers. Regardless of the cause for our inability to detect Brook Trout DNA in sediments, it is clear that either (1) bed sediments are incapable of sustaining Brook Trout DNA in headwater streams or (2) our sample handling and processing procedures were not capable of detecting Brook Trout DNA in streambed sediments. Until we can better understand the reasons for this quandary, sediments should not be considered an acceptable medium with which to detect eDNA and assess Brook Trout populations in headwater streams of the Adirondack region.

The present study faced several challenges due to the rapidly evolving nature of eDNA sampling and analysis methods. Foremost was the fact that the procedures used to collect and prepare stream water and sediment samples for use in fish eDNA analysis are not yet well standardized. Our initial collections relied on single (unreplicated) water and sediment samples. Although the lack of eDNA detections in sediment samples was noteworthy (as discussed above), the five false negatives from the water samples were problematic because they could cause potentially large errors in the inference of species distributions. Given that most studies sample or filter as little as 1 L (Blankenship et al. 2011) or 2 L (Jerde et al. 2011; Minamoto et al. 2012) of water, our choice to filter up to 6 L of water per sample (in accordance with the methods of Wilcox et al. 2013) was deemed sufficient for detecting relatively low concentrations of Brook Trout DNA in small headwater streams. Only after obtaining false negatives at five sites with low Brook Trout densities (generally <20 fish/0.1 ha) did we increase water volumes by doubling the number of field replicates that were collected at two of those sites. The results from duplicate eDNA samples from both sites were positive for Brook Trout DNA, indicating that either (1) up to 12 L of water should be filtered or (2) two or three filters should be pooled to ensure that relatively low eDNA concentrations (and low population densities) are appropriately detected.

Our findings have a number of important implications for the monitoring and assessment of Brook Trout populations (and entire fish assemblages) in streams of the Adirondack Mountains and elsewhere. First, our eDNA results indicated that in small headwater streams, water is a more effective sampling medium than streambed sediments. Whether sediments simply do not retain the genetic material eliminated from Brook Trout or our analytical methods were ineffective at detecting eDNA in sediments, most assessments of Brook Trout presence/absence and abundance in headwater streams should probably avoid the use of bed sediments until the issue is better understood. Second, the increased accuracy of eDNA results after the collection of replicate samples suggested that large sample volumes (filtered) should be considered either when the target species is expected to exist in low numbers or when the volume of occupied habitat is very large. Third, the ability of eDNA to accurately detect the presence of Brook Trout at very low densities (1–2 fish/100 m^2) means that the method is well suited for assessing population distributions across large regions and for assessing presence/absence at remote sites where gaining access with large crews and burdensome gear may be problematic. Fourth, the moderately accurate relationships between the relative eDNA concentration and Brook Trout density and biomass make eDNA an effective means of estimating population density and biomass. Although uncertainty (95% CIs) around the modeled lines was relatively low, the log scale made the uncertainty around actual predictions of population density or biomass for any given relative eDNA concentration quite large. Thus, the use of both models may be most appropriate for inferring the relative abundance (i.e., the absence or the relatively low, moderate, or high densities and biomass) of Brook Trout populations in streams of the Adirondack region. Quantitative fish surveys are often indispensable, however, for estimating the density and biomass of Brook Trout populations (and other species' populations) when the relationships between population (or community) metrics and predictor variables (e.g., stream discharge, chemistry, temperature, or toxicity) must be accurately characterized. Key objectives of research and monitoring studies or programs will dictate the quality and accuracy needed for fishery data and thus will determine whether the use of eDNA or the use of traditional survey methods is most appropriate. The scope of the project and funding levels for such efforts will also factor into these decisions. The fifth implication is that eDNA can provide a large cost benefit over traditional fish survey methods. Although sampling efforts vary widely with stream access, reach area, and fish abundance, the costs for an electrofishing

survey at a single stream site can range from US$500 to $3,000 (2016), whereas our experience and that of other groups (e.g., James Casey, Cornell University, personal communication) indicate that the costs for analyzing (not collecting and transporting) an eDNA sample could range from $20 to $50. Even if our per-sample collection and transportation costs (about $90) are factored into the estimate, there would still be a large cost benefit obtained from focusing on eDNA samples, especially for broad regional inventories of a single species at hundreds of sites.

Results from the present study and from other investigations point out a variety of limitations or issues with eDNA surveillance programs that need further study, development, or improvement. First, the reason behind the absence of (or our inability to detect) Brook Trout DNA in stream sediments is difficult to comprehend and should be further explored. Second, current eDNA monitoring efforts typically focus on a single aquatic species due to the limitations of the DNA amplification, isolation, and PCR quantification methods. It may now be possible to detect eDNA for three or four species from an individual eDNA sample by using PCR (Minamoto et al. 2012), and presence/absence information for common species can be obtained by next-generation DNA sequencing (Shendure and Ji 2008), but the inability of genomic methods to qualify abundance for more than a few species is a major deficiency. Metagenomic methodologies, which would enable us to detect all fish species at a site, are needed so that they could generate metrics for entire fish assemblages and other biotic communities from one eDNA sample or only a few eDNA samples. Third, additional investigations are also needed to devise, refine, or standardize effective eDNA sampling methods. Currently, the volumes of water filtered, the types of sampling devices employed (e.g., filters and centrifugation), and the time to collect eDNA samples can vary widely. Fourth, the variability in the number of gene sequences in water samples is not well understood; thus, sample volumes, the number of field replicates, and the number of laboratory replicates may be larger than necessary. More information on the persistence of eDNA under different environmental conditions and in different types of surface water could help to standardize sampling and analysis procedures. Fifth, our knowledge of the environmental factors that affect eDNA persistence or degradation in the field and inhibit DNA amplification in the laboratory has increased steadily over the past decade but is still far from complete. Most of the information gaps and technical challenges that remain should be addressed in the not-so-distant future given the rapidly evolving field of eDNA analysis and its enormous potential to change the way in which field studies monitor and assess species' populations, communities, and entire ecosystems.

ACKNOWLEDGMENTS

We extend our appreciation to Noel Deyette, Luis Rodriguez, and Nick McCloskey (USGS); Howard Simonin (New York State Department of Environmental Conservation, retired); and Spencer Bruce (State University of New York at Albany) for field support. This research was funded by the New York State Energy Research and Development Authority and the USGS. Any use of trade, firm, or product names is for descriptive purposes only and does not imply endorsement by the U.S. Government.

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