J. Great Lakes Res. 34:228–234
Internat. Assoc. Great Lakes Res., 2008

Sources and Sinks of *Escherichia coli* in Benthic and Pelagic Fish

Dennis L. Hansen[1], John J. Clark[1], Satoshi Ishii[2], Michael J. Sadowsky[2,3], and Randall E. Hicks[1,*]

[1]*Department of Biology*
University of Minnesota Duluth
207 Swenson Science Building
1035 Kirby Drive
Duluth, Minnesota 55812-3004

[2]*Department of Soil, Water, and Climate*
[3]*BioTechnology Institute*
University of Minnesota
439 Borlaug Hall, 1991 Upper Buford Circle
St. Paul, Minnesota 55108

ABSTRACT. Escherichia coli *and fecal coliform bacteria were isolated from five benthic and four pelagic fish species to determine their role in the fecal contamination of recreational waters. All fish were collected during fall 2006 from Southworth Marsh in the Duluth-Superior Harbor, a public beach that is commonly posted to minimize water contact due to high* E. coli *levels. Although fecal coliform bacteria were isolated from each fish species, they were only isolated from 66% and 72% of the individual benthic and pelagic fish, respectively. While 42% of the fecal coliforms from benthic fish were* E. coli, *only 4% of these bacteria from pelagic fish were* E. coli. *Cluster analysis showed different fish species harbored identical strains of* E. coli *and some fish contained multiple* E. coli *strains. The potential source for 65% of the* E. coli *isolates obtained from fish were identified by using the HFERP DNA fingerprinting method and libraries of* E. coli *DNA fingerprints from warm-blooded animals and environmental isolates collected in the area. The* E. coli *strains whose source could be identified were most similar to strains isolated from sediments, Canada geese, mallard ducks, and wastewater. None of the fish* E. coli *had DNA fingerprints matching those from any water or beach sand isolates. Although our results demonstrate that benthic fish contain* E. coli*, it may be more appropriate to consider these fish as a vector of* E. coli *from other sources, rather than a new source of* E. coli *contamination in aquatic environments.*

INDEX WORDS: *Fecal coliforms,* E. coli*, benthic fish, pelagic fish, sediment, microbial source tracking.*

INTRODUCTION

The presence of *Escherichia coli* is currently used as an indicator of recent fecal contamination in recreational waters. There are many possible sources of *E. coli* in the environment, including waterfowl (Alderisio and DeLuca 1999, Ishii *et al.* 2007, Jones *et al.* 1978, Levesque *et al.* 2000, Standridge *et al.* 1979), wildlife (all animals except waterfowl) (Ishii *et al*. 2007, Johnson *et al.* 2004, Vogel *et al.* 2007), algae and periphyton (Ishii *et al.* 2006b, Ksoll *et al.* 2007, Whitman *et al*. 2003), soils and sediments (Byappanahalli *et al.* 2006, Ishii *et al.* 2006a, Ishii *et al.* 2007), and treated wastewater effluent (Ishii *et al.* 2007, USEPA 1986). Wild fish, however, are relatively understudied and often an overlooked potential source of *E. coli*. There have been several studies on the presence of fecal coliforms (FC) in farm-reared fish because of concern about the health of fish consumers. Molinari *et al*. (2003) reported that bacteria in the genera *Aeromonas, Burkholderia, Chromobacterium, Citrobacter, Escherichia, Flavimonae, and Plesiomonas* are present in farm-raised tilapia. Del Rio-Rodriguez *et al.* (1997) used *E.*

*Corresponding author. E-mail: rhicks@d.umn.edu

coli-laden feed to infect rainbow trout (*Oncorhynchus mykiss*) intestines and Al-Harbi (2003) detected *E. coli* in farm raised tilapia intestines and correlated them with pigeon droppings.

In contrast, there have been relatively few studies about the presence of fecal coliforms and *E. coli* in wild fish. Geldreich and Clarke (1966) showed that fecal coliforms and *Streptococcus faecalis* could be found in fish intestines. They speculated that fecal coliforms are not part of the permanent microflora in fish, but their presence is caused by polluted water and is influenced by feeding habits. Guzman *et al.* (2004) found two fish species that harbored *E. coli* from a river contaminated by sewage effluent. Their work also supported the view that fish obtain *E. coli* from the environment. None of the aforementioned studies attempted to trace the sources of *E. coli* in these fish using any technique. Microbial source tracking techniques (Santo Domingo and Sadowsky 2007), which have been used to identify the sources of environmental *E. coli*, can also be used to identify the sources of *E. coli* in fish. Knowing these sources would allow us to better understand the role, if any, that fish play in microbial contamination of aquatic habitats.

To trace the sources of *E. coli* isolated from wild fish, we used the horizontal fluorophore enhanced rep-PCR (HFERP) DNA fingerprinting technique, as described by Johnson *et al.* (2004) and Ishii *et al.* (2007). The HFERP method compares *E. coli* DNA fingerprints of interest to a library of fingerprints from known sources, and has been successfully used to identify the probable source of unknown environmental *E. coli* strains (Byappanahalli *et al.* 2006, Ishii *et al.* 2007, Ksoll *et al.* 2007). The objectives of our study were to [i] determine if fecal material from fish in the Duluth-Superior Harbor contained fecal coliforms and *E. coli*, and [ii] determine if *E. coli* found in fish fecal material are primarily from animal or environmental sources.

METHODS

Site Description

Southworth Marsh (46°44′26.370″N, 92°03′42.804″W) is a public beach in the Duluth-Superior Harbor in Duluth, MN that is monitored for *E. coli* contamination by the Minnesota Pollution Control Agency (MPCA, http://www.mnbeaches.org/beaches/lksuperior/B036.shtml). The site is a popular recreation area for sailing, kayaking, and rowing, and is frequently visited by mallard ducks (*Anas platyrhynchos*) and Canada geese (*Branta Canadensis*). Signs not recommending water contact are frequently posted at the Southworth Marsh beach by the MPCA in the summer and fall due to high *E. coli* levels.

Fish Collection and *E. coli* Verification

Fish were collected at Southworth Marsh with fyke nets from August through October 2006. The benthic species collected were the common carp (*Cyprinus carpio*; n = 6), white sucker (*Catostomus commersonii*; n = 6), brown bullhead (*Ameiurus nebulosus*; n = 9), ruffe (*Gymnocephalus cernuus*; n = 3), and round goby (*Neogobius melanostomus*; n = 8). The pelagic species were white perch (*Morone Americana*; n = 10), yellow perch (*Perca flavescens*; n = 3), pumpkinseed (*Lepomis gibbosus*; n = 6), and rock bass (*Ambloplites rupestris*; n = 6).

Fecal coliform (FC) bacteria were isolated from fecal material of these five benthic and four pelagic species (Table 1). After collection, the fish were transported in harbor water to the lab where their intestines were aseptically removed. Fecal material was extracted from intestines and plated onto mFC agar (Difco, Detroit, MI) to isolate fecal coliform bacteria. The isolation and verification of *E. coli* were completed as described by Ishii *et al.* (2006a), with minor changes. After overnight incubation at 44.5°C, colonies that were dark blue on mFC agar were transferred to MacConkey Agar (Difco). Pink and red isolates on MacConkey agar were tested on CHROMagar ECC (CHROMagar Microbiology, Paris, France). Bacterial colonies that were white or blue on CHROMagar ECC were verified to be *E. coli* as described by Ishii *et al.* (2006a). Isolates testing positive as *E. coli* were stored in a 50% glycerol solution in CryoTube™ vials (Nunc, Roskilde, Denmark) at –80°C until DNA fingerprints were obtained by using the HFERP DNA fingerprinting method (Johnson *et al.* 2004).

HFERP Analysis and Host Library

Fish isolates were initially compared to a host library of HFERP DNA fingerprints of *E. coli* from animals and wastewater collected from northeastern Minnesota. The host *E. coli* library consisted of 955 unique DNA fingerprints from 8 sources, including herring gulls (*Larus argentatus*; 4 fingerprints), beaver (*Castor Canadensis*; 10), deer (*Odocoileus virginianus*; 52), ducks (72), common terns (*Sterna hirundo*; 91), Canada geese (139), ring-billed gulls

TABLE 1. Pelagic and benthic fish sampled from Southworth Marsh in 2006. This table shows the percentages of fish species harboring fecal coliforms,* E. coli*, and the relative abundance of* E. coli *in each fish species.

Pelagic Fish	Number Sampled	Fish with Fecal Coliforms (%)	Fecal Coliforms Tested (number)	Fish with *E. coli* (%)	*E. coli* isolates (number)
Yellow Perch	3	100	27	0	0
Pumpkinseed	6	67	20	0	0
White Perch	10	50	6	10	1
Rock Bass	6	100	30	17	2
Total	25	72	83	8	3
Benthic Fish					
Brown Bullhead	9	56	26	22	24
Ruffe	3	67	1	33	1
Round Goby	8	38	3	25	2
White Sucker	6	100	18	0	0
Common Carp	6	83	19	17	1
Total	32	66	67	19	28

(*Larus delawarensis*; 247), and treated wastewater (340). *E. coli* strains were isolated from the Western Lake Superior Sanitary District's (WLSSD) sewage treatment facility, which is 4.5 km from Southworth Marsh and discharges effluent into the Duluth-Superior Harbor. Fingerprints from fish *E. coli* that could not be matched to an animal host were then compared to a library of 1,163 DNA fingerprints from environmental *E. coli* isolates. This environmental *E. coli* library contains strains from water, beach sand, and sediments, collected from Southworth Marsh during 2005 and 2006.

Cluster analysis, and bootstrap identification of the fish *E. coli* fingerprints were assessed by using BioNumerics software (Applied Maths, Sint-Martens-Latem, Belgium) as described by Ishii *et al.* (2006a) and Johnson *et al*. (2004). A dendrogram was created using the unweighted pair group method with arithmetic means (UPGMA) and Pearson's product-moment correlation coefficient (Ishii *et al*. 2006a, 2007; Johnson *et al*. 2004). Only fingerprints that matched a source in the host or environmental libraries with a $p \geq 0.90$ were considered a match (Byappanahalli *et al*. 2006, Ishii *et al*. 2007).

RESULTS

Culturable fecal coliforms were found in each fish species, but only 66% of the benthic fish and 72% of the pelagic fish contained fecal coliform bacteria (Table 1). The percentage of benthic fish harboring fecal coliforms was not statistically different ($p > 0.1$) than the percentage of pelagic fish containing fecal coliforms. On average, only 19% of the benthic fish examined contained *E. coli*, which was not different from the pelagic fish species (average of 8%; $p > 0.1$). However, the percentage of fecal coliforms isolated from benthic fish that were verified as *E. coli* (42%) was 10 times greater than for the pelagic fish species examined (4%; $p < 0.10$).

We considered *E. coli* isolates to be clones (i.e., identical strains) if the similarity of their HFERP DNA fingerprints was ≥ 92% (Ishii *et al.* 2006a, Ishii *et al.* 2007). *E. coli* strains isolated from all fish clustered into 12 strain groups using this criterion (Fig. 1). ID bootstrap analysis identified the probable origin of *E. coli* in nine of these strain groups, and 65% of the *E. coli* isolated from all fish ($n = 31$) likely originated from animal or environmental sources. Almost all of these *E. coli* were obtained from benthic fish (18 of 20 strains), primarily from brown bullheads.

Overall, *E. coli* strains from all benthic and pelagic fish combined, whose source could be identified, were most similar to *E. coli* found in beach sediments (8 isolates), Canada geese (6 isolates), mallard ducks (3 isolates), and wastewater (3 isolates). Most of the *E. coli* strains identified to be from beach sediments were found in brown bull-

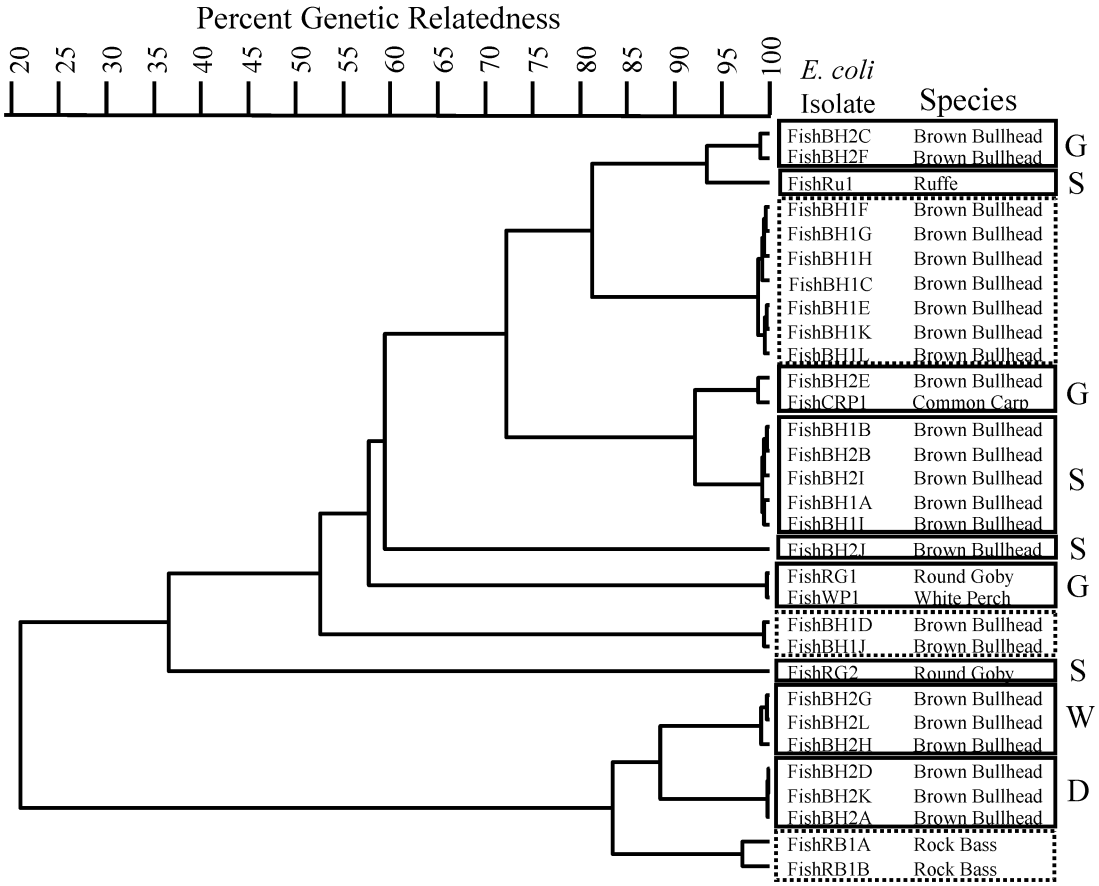

FIG. 1. Dendrogram showing the genetic similarity of* E. coli *isolated from different fish and the probable source of these* E. coli*. The* E. coli *isolates in solid-line boxes at the right were from the same source. These sources are denoted to the right of each box: S = sediment, G = Canada geese, D = mallard ducks, and W = wastewater* E. coli*. Boxes with dotted-lines indicate groups of fish* E. coli *whose source could not be identified using the HFERP method and the animal and environmental source libraries. The scale bar represents percent similarity of the HFERP DNA fingerprints of each strain.

heads, although sediment *E. coli* strains were also found in two other benthic fish species, ruffe and the round goby (Fig. 1). The *E. coli* strains from Canada geese were found in brown bullheads, common carp, round goby, and white perch. A round goby and white perch fish shared an *E. coli* strain originating from Canada geese. Brown bullheads were the only fish that contained *E. coli* from mallard ducks and wastewater. The two *E. coli* strains isolated from the pelagic rock bass were identical, but the source of this strain group could not be identified using our animal and environmental source DNA fingerprint libraries.

Most of the *E. coli* strains that could be identified were obtained from brown bullhead, but this was not surprising considering that most *E. coli* were isolated from this species (Table 1). The brown bullheads harbored a variety of *E. coli* strains (Fig. 1), and isolates from one of these fish were found in three different *E. coli* strain groups. An animal or environmental source for two of these strain groups could not be identified, but the third strain group was most similar to *E. coli* isolated from beach sediments. This *E. coli* strain was also found in another brown bullhead that also harbored *E. coli* strains from five other strain groups, which were most similar to Canada geese (2 clusters), mallard ducks (1 cluster), and wastewater (1 cluster).

DISCUSSION

The presence of fecal coliform bacteria in the intestinal tracts of fish that inhabit polluted waters is well documented (Al-Harbi 2003, Del Rio-Rodriguez *et al.* 1997, Stanistaw and Tucholski 2000, Geldreich and Clarke 1966, Guzman *et al.* 2004, and Trust 1975). Fecal coliforms in fish are influenced by fish feeding habits, and fish can harbor fecal coliforms up to 14 days after being exposed to contaminated water (Geldreich and Clarke 1966). In our study, fecal coliforms were found in the fecal materials of every fish species examined, but not every fish. This inconsistency supports Geldreich and Clarke's (1966) view that fish may not harbor stable populations of fecal coliforms, but rather acquire allochthonous microorganisms in conjunction with feeding activity.

Geldreich and Clarke (1966) also found that benthic fish species harbored more fecal coliforms than pelagic species. Our results are consistent with their observation. Although the percentages of benthic and pelagic fish species harboring fecal coliforms or *E. coli* were not significantly different, the percentage of fecal coliforms verified to be *E. coli* in benthic fish was 10 times larger than the percentage in pelagic fish species. It might be expected that benthic fish would harbor more *E. coli* than pelagic fish because *E. coli* are less abundant in water than in sediments (Desmerais *et al*. 2002, Ishii *et al.* 2007, LaLiberte and Grimes 1982, Obiri-Danso and Jones 2000), where benthic species often feed. The digestive tracts of benthic fish are probably inoculated with fecal coliforms from the sedimentary organic material they consume (Del Rio-Rodriguez *et al.* 1997, Trust 1975). We recognize that if brown bullheads had not been sampled, then the percentage of fecal coliforms verified to be *E. coli* in benthic fish would be more similar to the pelagic fish species examined. Brown bullheads may not be representative of all benthic fish species. Clearly, additional studies are needed to more thoroughly document differences in the numbers of fecal coliforms and *E. coli* found in benthic and pelagic fish species.

An animal or environmental source could be identified for 65% of all *E. coli* isolated from fish fecal materials used in this study. The remaining 35% of the *E. coli* may come from sources not represented in our animal or environmental sources libraries, or possibly some *E. coli* strains may have become naturalized to fish. Unfortunately, our results cannot be used to determine which of these cases may be true for the unidentified strain groups. If any of the unidentified strains could be repeatedly isolated from the fecal materials of the same fish species over a longer period than that used in our study, then it may be possible to determine if the unidentified strains were naturalized to fish intestinal tracts using the criteria defined by Ishii *et al*. (2006a), Ishii *et al*. (2007), and Ksoll *et al.* (2007).

Of the *E. coli* isolates from fish fecal materials whose source could be identified, 60% were from various animal hosts and the remaining 40% likely originated from sediments. Even though most of the *E. coli* were isolated from benthic fish species, it is interesting that none of them matched environmental *E. coli* strains obtained from water or beach sand at Southworth Marsh. Whitman *et al*. (2004) showed that exposure to sunlight can inactivate *E. coli* in water. Sediments, where benthic fish often feed, contain higher levels of *E. coli* than water (Obiri-Danso and Jones 2000, Ishii *et al*. 2007), and sediment materials probably provide bacteria some protection against light-induced inactivation. Considering these facts, it appears less likely that pelagic fish will ingest or be inoculated by as many *E. coli* as benthic fish.

Canada geese, mallard ducks, and wastewater were the next most common sources of *E. coli* found in benthic fish. Canada geese and mallard ducks regularly forage and sleep at Southworth Marsh, and approximately 45 million gallons of treated wastewater are discharged into the Duluth-Superior Harbor each day (http://www.ci.superior.wi.us/index.asp?nid=83, www.wlssd.com/wastewater_treatmentprocess.php). *E. coli* from waterfowl and treated wastewater have been found to settle onto sediments (Ishii *et al.* 2007), where they may be consumed by benthic fish. However, other studies have shown that *E. coli* can survive for long periods and some strains may become naturalized to soils and periphyton (Gordon *et al.* 2002, Whitman *et al*. 2003, Ishii *et al.* 2006a, Ksoll *et al.* 2007). Naturalized strains of *E. coli* could also persist in fish. It is clear from our data, however, that the fecal material from at least one benthic fish species (e.g., brown bullheads) was not dominated by a single *E. coli* strain, but rather contained many strains from multiple animal and environmental sources. Certainly, additional studies having more *E. coli* isolates for each fish species than we obtained could help confirm this finding. Still, it is probably more appropriate to consider the fish species in this study as vectors of *E. coli* from other

sources rather than a new source of *E. coli* contamination in waterways, considering the small percentage of fish in each species that contained *E. coli* and the multiple sources of *E. coli* found in brown bullheads.

ACKNOWLEDGMENTS

We thank the United States Environmental Protection Agency, Mid-Continent Ecology Division of the National Health and Environmental Research Laboratory, in Duluth, MN for loaning us their nets. We also thank Jenna Bergin, Jason Kish, John Gralewski, William Rusk, and Ryan Oster for providing technical assistance in the field and the lab.

This work was supported by the University of Minnesota Undergraduate Research Opportunities Program (to J. J. C.) and by a grant from the Minnesota Sea Grant College Program, NOAA Office of Sea Grant, United States Department of Commerce, under grant no. NA03-OAR4170048 (to R.E.H. and M.J.S.). The U.S. Government is authorized to reproduce and distribute reprints for government purposes, not withstanding any copyright notation that may appear hereon. This paper is journal reprint No. JR. 544 of the Minnesota Sea Grant College Program.

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Submitted: 17 July 2007
Accepted: 23 November 2007
Editorial handling: William C. Sonzogni