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Environmental impact statement:

F-specific RNA coliphages were identified from human-impacted locations in surface waters during winter months. Data on the prevalence of F-specific RNA coliphages may be invaluable in predicting the sources of fecal contamination of waters used for produce cultivation and aid in designing methods to prevent enteric pathogens in irrigation waters.

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ARTICLE TYPE

Male-specific coliphages for source tracking fecal contamination in surface waters and prevalence of Shiga-toxigenic *Escherichia coli* in a major produce production region of central coast of California

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To provide data for traditional trace-back studies from fork to farm it is necessary to determine the environmental sources for Shiga-toxigenic *Escherichia coli*. We developed SYBR green based reverse-transcriptase PCR methods to determine the prevalence of F+ RNA coliphages (FRNA) as indicators of fecal contamination. Male-specific coliphages, determined using single-agar overlay method, were prevalent in all surface waters sampled during 8 months. F+ DNA coliphages (FDNA) were predominant compared to FRNA in waters from majority of sampling locations. Most (90%) of the FRNA were sourced to humans and originated from human-impacted sites. Members of genogroup III represented 77% of FRNA originated from human sources. Furthermore, 93% of FRNA sourced to animals were also detected in waters from human-impacted sites. Eighty percent of all FRNA were isolated during the winter months indicating seasonality in prevalence. In contrast, FDNA were more prevalent during summer months. *E. coli* O157:H7 and Shiga-toxigenic *E. coli* were detected in water samples from locations predominantly influenced by agriculture. Owing to the scarcity, their numbers could not be correlated with the prevalence of FRNA or FDNA in waters. Both coliform bacteria and generic *E. coli* from agricultural or human-impacted sites were similar in numbers and thus could not be used to determine sources of fecal contamination. Data on the prevalence of male-specific coliphages may be invaluable in predicting the sources of fecal contamination and aid in developing methods to prevent enteric pathogen contamination from likely sources during produce production.

INTRODUCTION

Source-tracking *Escherichia coli* O157:H7 (EcO157) and other Shiga-toxigenic (STEC) *E. coli* is critical to aid in preventing their spread from point sources, such as feedlots and dairies, to fruit and vegetable crops grown in proximity. Indeed, EcO157 isolates, genetically indistinguishable from the 2006 spinach outbreak strain, were isolated from feces from feral swine and cattle, water and sediment samples from creeks near spinach fields in the central coast region of California.[1] Traditionally, source-tracking of the target pathogen is done after an outbreak has occurred using labor-intensive samplings[2] from the environment, followed by enrichment culturing, magnetic bead based separations coupled with isolations on selective media and characterizations using molecular methods[3]. Furthermore, trace-back studies after an outbreak are often unsuccessful, as pathogen levels are often transient in the environment.

Since dairy and feedlot cattle are either sources or carriers of EcO157 and other STEC *E. coli*, determination of the sources and transport of fecal contamination appears to be a less resource intensive alternative to tracking the target pathogen after an outbreak. Sources of fecal contamination were previously determined using chemical and biomarkers.[4-7] Twenty-six microbial and chemical tracers were used in a multi-laboratory study[8] to determine fecal sources. They determined that 'no one source identifier alone provided 100% correct classification of the fecal source. However, the study indicated that male-specific coliphages (MSC) as potentially useful source indicators of fecal contamination. Nevertheless, once fecal sources are identified, it would be prudent to determine the prevalence and abundance of the target pathogen at the predicted source.

MSC infect only F+ male *E. coli* host cells through the F sex pilus[9] and consists of F+ DNA coliphages (FDNA) and F+ RNA coliphages (FRNA). FDNA are single-stranded DNA phages of family *Inoviridae* and FRNA are single-stranded RNA phages of family *Leviviridae*. FRNA are clustered into four genogroups based on their serological cross-reactivity, replicase template activity and sequence phylogeny.[10] Genogroups I and IV are generally associated with animals, and those of genogroups II and III are associated with humans.[11, 12]

FDNA were characterized from raw sewage,[13] surface waters from agricultural watersheds,[14] municipal wastewaters, bovine and swine wastewaters.[9] FRNA were characterized from ground and surface waters,[14, 15] raw and treated sewage,[13, 16] estuarine oysters[6] suspected of fecal contamination and fecal samples from various animals[17]. Human fecal contamination was

predicted in surface waters from Kofu river basin in Japan based on the high abundance of genogroup II FRNA in waters[16]. Likewise, fecal inputs from a nearby hobby farm were suspected based on elevated loads of FDNA and FRNA in waters from agricultural watersheds[14].

Tracking FDNA and FRNA of distinct genogroups would be beneficial in determining the likely host sources of STEC *E. coli* in surface waters. Thus, in the current study MSC were characterized along with coliform bacteria, EcO157, and other STEC *E. coli* from water samples from Salinas River and its associated tributaries in California. These sampling sites included agricultural locations and additional sites likely impacted by human activities within the city of Salinas. MSC were initially isolated using male-specific *E. coli* as host in a single-agar overlay technique and further characterized into FDNA and FRNA based on RNase sensitivities. FRNA were then characterized as belonging to one of the four genogroups by real-time reverse-transcriptase PCR (RT-PCR) techniques. Spatial and temporal changes in populations of MSC, STEC *E. coli* and coliform bacteria during an 8-month period were also monitored to determine the relationship between the prevalence of MSC and the incidence of pathogenic *E. coli* in produce production environments.

MATERIALS AND METHODS

Water and swab samples

Water (500 ml) and Moore swab samples were collected during December 2012 to August 2013, as detailed by Cooley *et al*.,[3] at two different locations in Salinas River and four locations in or near the city of Salinas (Fig. 1; Table 1). These last four locations are fed from the Gabilan and Alisal Creeks and eventually drain to the Espinosa Slough. Moore swabs were collected 24 hrs after anchoring them in water sources. Three locations were chosen specifically for their proximity to municipal treatment facilities and human encampments (circle in the inset, Fig. 1) alongside the creeks. Samples were transported on ice to the laboratory. Upon arrival the Moore swabs were shaken briefly with 400 ml of sterile water and sub-samples were taken from both river waters and swab extracts. Water samples were used for enumeration of coliform bacteria, MSC, FDNA and FRNA. Swab extracts were used for enumeration of *E. coli,* MSC, FDNA and FRNA, and detection of STEC *E. coli* and EcO157. A total of 84 water samples and 84 swab extracts were processed.

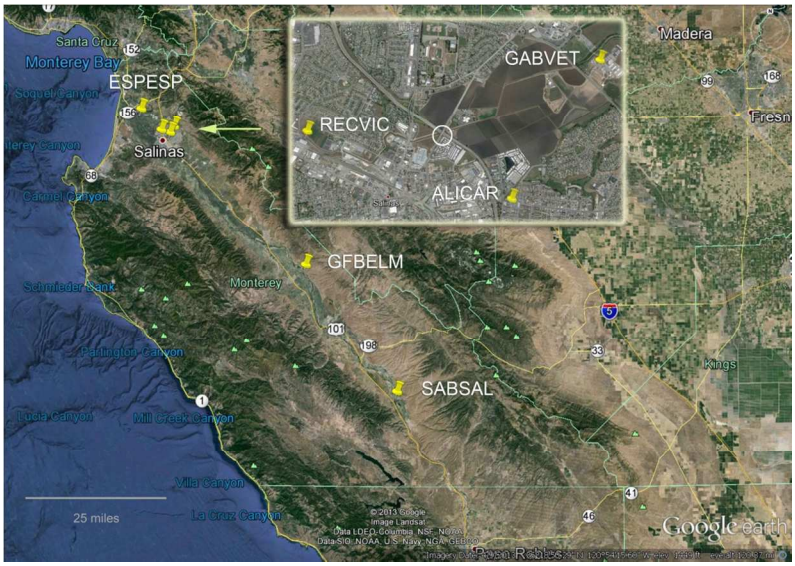

Fig. 1 Sampling locations along the waterways and creeks feeding into Salinas River in central coast of California. Inset shows sampling locations impacted by human activities

Table 1 Water and swab sampling locations

Location code	Location	Details	GPS coordinates, decimal degrees	
			Latitude	Longitude
ESPESP	Espinosa Slough at Espinosa Road, Salinas, CA	Reclamation ditch near and downstream of agricultural fields	36.73988473	-121.73265368
RECVIC	Reclamation ditch at Victor Street, Salinas, CA	Near reclamation canal. Gabilan and Alisal creeks feed into the canal. Downstream from Carr Lake[a] and	36.68406599	-121.66718732

		human encampments		
GABVET	Gabilan Creek inlet to Carr Lake, Salinas, CA	Feeds into Carr Lake and is upstream to human encampments	36.69287795	-121.62694725
ALICAR	Alisal Creek inlet to Carr Lake, Salinas, CA	Downstream to human encampments	36.67736553	-121.63945327
GFBELM	Salinas River at Elm Avenue, Greenfield, CA	Agricultural location	36.33878342	-121.20454667
SABSAL	Salinas River at Cattlemen Road, San Ardo, CA	Agricultural location	36.01560005	-120.91629160

[a]During the late 18th century, Carr Lake and the surrounding swamps were drained and reclaimed for agriculture (http://www.mchsmuseum.com/salinasbrief.html). Occasional flooding at this location is common.

Enumeration of coliform bacteria and isolation of STEC *E. coli* and EcO157

Coliform bacteria from 10-fold serial dilutions of water samples were analyzed using 1-ml dilutions on *E. coli*/coliform petrifilm count plates (3M Microbiology Products, St. Paul, MN). The enumeration plates were incubated overnight at 37°C and the red and blue colonies associated with gas bubbles were counted separately to confirm coliforms and *E. coli*. *E. coli* numbers in water extracts of swabs were determined by the Colilert QuantiTray 2000 method according to the manufacturer's instructions (Idexx Laboratories, Westbrook, ME).

STEC *E. coli* and EcO157 were isolated from enrichments of swab samples and characterized using cultural, molecular and immunological methods described earlier[2].

Enumeration and culturing MSC

MSC from river water and swab samples were enumerated by spotting ten-fold serial dilutions on a lawn of *E. coli* host strain C3000[18]. LB agar plates were coated with overnight growth of *E. coli* host by layering the agar surface with 1 ml cells of OD_{600} 0.3 and siphoning-off the excess cell suspension with a pipette. The plates were allowed to incubate for 3-4 hours prior to inoculation of water samples.

Five *E. coli* coated plates each were inoculated with 10 spots of 10-µl portions of each sequential dilution (100 µl x 5) and the plates were incubated overnight at 37°C. Phage plaques from the highest dilutions were counted. Agar plugs from all plaques from this dilution were individually transferred using 1 ml sterile wide-bore pipette tips to 2 ml sterile snap-cap vials containing 100 µl over-night growth of C3000 in PBS (OD_{600} 0.3). The vials were incubated at 37°C for 24 h and then stored at 4°C until further characterization.

Phage recovery method was validated using two different water samples spiked with reference FRNA. Water samples previously tested as negative from RECVIC and ALICAR were spiked in triplicates with 10^2 and 10^3 PFU/ml of FRNA (MS2, GA, QB, and SP), the dilutions of waters were then spotted onto C3000-coated LB agar plates and the plaques were enumerated as described above.

MSC were further subjected to RNase sensitivity to differentiate FRNA from FDNA. Isolated MSC were spotted on the surface of C3000-coated LB agar supplemented with 80 µg/ml of RNase[19] (Sigma-Aldrich, St. Louis, MO) and the plaques were counted as FDNA.

Characterization of FRNA by RT-PCR

FRNA were regrown overnight at 37°C in 700 µl of freshly grown host cells and checked for purity from ten-fold dilutions spotted on C3000-coated LB-agar plates. FRNA from triplicate clearing zones from the highest dilution were re-grown overnight in LB-broth containing C3000 and used for viral RNA extraction. Phage (140 µl) was extracted using QIAamp Viral RNA Mini Kit (Qiagen Sciences, Germantown, MD) as per manufacturer's instructions. Viral RNA was eluted in 60 µl of RNase-free buffer and used for RT-PCR.

FRNA were characterized into 4 different genogroups by RT-PCR using genogroup specific primers designed by Wolf *et al.*[20] and synthesized by Integrated DNA Technologies (Coralville, IA). QuantiFast SYBR green RT-PCR kit (Qiagen) was used for the assays. Each 25-µl reaction mixture contained 12.5 µl 2x QuantiFast RT-PCR master mix, 250 nM each of forward and reverse primers, 5 µl template, 2.25 µl RNase-free PCR-grade ultrapure water and 0.25 µl QuantiFast RT mix containing Omniscript and Sensiscript reverse transcriptases. Separate RT-PCR reactions were carried out for each genogroup. Duplicate template-free controls and reverse transcriptase-free template controls for each reference FRNA were included for amplification in a Stratagene MX3000P QPCR system (Agilent Technologies, Inc., Santa Clara, CA). Thermal cycling parameters were 95°C for 5 min denaturation, annealing and extension at 95°C for 10 s over 40 cycles and 60°C for 30 s. Three replicate assays were performed for each purified phage RNA sample.

FRNA strains MS2, GA, QB and SP, kindly provided by Prof. M. Sobsey of University of North Carolina, were used as references for genogroups I, II, III and IV, respectively. MxPro QPCR software was used to characterize FRNA by using C_T (threshold cycle) values. Dissociation curves were used to eliminate ambiguities in characterization of FRNA.

RESULTS

Recovery of FRNA from spiked river water samples

Phage recoveries, by the single-agar layer method, at two spiking levels ranged between 50 to 72% for reference strains inoculated to waters from RECVIC and ALICAR (Table 2).

Table 2 Validation of quantitative recoveries from spiked water samples

Reference strain (genogroup)	Spike level, PFU/ml (x10)	Percent recovery of spiked phages	
		RECVIC	ALICAR
MS2 (GI)	173 ± 0.5	64.7 ± 0.7	59.2 ± 0.4
	17.3 ± 0.05	74.0 ± 2.9	63.6 ± 2.0
GA (GII)	150 ± 0.3	63.6 ± 0.8	64.8 ± 0.6
	15.0 ± 0.03	50.7 ± 1.4	72.0 ± 1.8
QB (GIII)	132 ± 0.1	69.2 ± 0.7	70.6 ± 0.7
	13.2 ± 0.01	63.6 ± 1.8	56.1 ± 1.1
SP (GIV)	147 ± 0.2	62.4 ± 0.2	62.4 ± 0.6
	14.7 ± 0.02	50.3 ± 1.3	58.5 ± 1.2

Single-agar layer method was chosen in preference to the double-agar layer method for the presence of distinct plaques on the C-3000-coated agar surface. Non-discernible and diffused plaques were obtained with the double layer method.

Characterization of FRNA by SYBR green RT-PCR

Probe-based methods[20] were modified for characterization of each FRNA separately using SYBR green one-step real-time RT-PCR. Limits of detection were C_T value minus two standard deviations from template-free controls. Minimum detectable limits were calculated to be between 0.9 to 2.1 PFU per assay (1.8 to 4.2 x 10^2 PFU per ml water) for MS2, GA, and QB and 36 PFU for (7.2 x 10^3 PFU per ml) SP. Purified FRNA isolated from waters were characterized qualitatively as belonging to one of the four genogroups.

Enumeration and characterization of MSC from water samples

MSC were detected from water and swabs collected from all sampling locations during an 8-month period (Tables 3 and 4; Fig. 2). A total of 244 PFUs were isolated from both water and swab samples (Table 3) and 67% of them were characterized as FDNA by RNase sensitivity assay. FDNA were predominant compared to FRNA from all sampling locations except ALICAR. PFUs of FDNA increased steadily during the months of May to August 2013 in samples from Espinosa Slough (Fig. 1 and 2). In addition, all of MSC were characterized as either FDNA or FRNA.

Of the FRNA, 94% were characterized as belonging to one of four genogroups. Most FRNA were from ALICAR and RECVIC, which also yielded highest levels of FRNA originated from humans. Samples from GABVET, GFBELM and SABSAL did not contain any known FRNA from animals. FRNA from either humans or animals were isolated from 5 out of 6 locations and 76% of them were from human sources (Table 3). Of the FRNA from human sources, 76% matched with reference strain QB.

Populations of FRNA also varied widely with season (Table 4). FRNA from human-impacted sources were more prevalent in samples collected during the months of January to March of 2013, while no such trends were observed for FRNA from animal sources. However, FDNA were more predominant in waters collected during the months of April to August (Table 4). Prevalence of FDNA or FRNA did not correlate with rain events but it rained during the weeks QB-like FRNA were detected (Table 4).

FDNA, FRNA, *E. coli* O157, STEC *E. coli,* generic *E. coli,* and coliform bacteria

Populations of FDNA and FRNA were plotted by sample date (Fig. 2) along with coliform bacteria in water and *E. coli* from Moore swabs to determine if any relationship can be established between fecal contamination and prevalence of EcO157 and STEC *E. coli* (Table S1). STEC *E. coli* were detected from 4 out of 6 sampling locations and EcO157 were detected from 3 out of the same 4 locations.

Rainfall within two weeks prior to samplings did not influence the total numbers of MSC (Table 4) or prevalence of STEC *E. coli* or ECO157 (Fig. 2) but significantly influenced (Pearson's $r = 0.847$; $P < 0.0001$) the populations of FRNA originated from humans at location RECVIC. Seasonal fluctuations in MSC or FDNA could not be correlated also with the numbers of *E. coli,* coliform bacteria or STEC *E. coli*.

A total of 23 STEC *E. coli* and 8 EcO157 strains were characterized from swab samples (Table S1). Majority of them (71%) were from two of the agricultural locations, GFBELM and SABSAL (Fig. 2). EcO157 were found in only 5 out of 42 samples collected from three locations (ALICAR, GFBELM and SABSAL; Fig. 2). Most positives for EcO157 were found in 3 swabs collected during January 2nd, 2013 to February 21st, 2013 from GFBELM and most positives for STEC *E. coli* were from SABSAL.

Coliform bacteria in waters fluctuated between 0.3 to 3.8 log CFU/ml, whereas *E. coli* in swabs ranged from 2.8 to 6.6 log CFU/ml (Fig. 2). *E. coli* numbers were expectedly high as the swabs accumulate organisms during a 24 h period compared to coliform concentrations in free-flowing waters. Furthermore, *E. coli* numbers could not be related to the incidence of EcO157 and STEC *E. coli* monitored also from swabs.

DISCUSSION

FRNA are often used as source identifiers for fecal contamination,[4, 9, 15, 16, 21, 22] and thus we investigated if contamination had occurred in surface waters near produce production regions of California. We monitored the surface waters initially for the prevalence of MSC using a modified single-agar overlay method. Our findings indicated that MSC were highly prevalent in surface waters and were detected from water and swab samples collected from all locations during the 8-month period. Interestingly, 67% of all MSC were characterized as FDNA and were found at most sampling locations and sampling periods (Tables 3 and 4). FDNA were predominant in waters influenced by both agricultural and human activities. Similarly, FDNA were found in abundance compared to FRNA in surface waters by Cole *et al.*[9] and were also linked with cattle and human-impacted sites. FDNA were also found in raw sewage but not in secondary treated sewage[13]. In contrast, only 5% phage plaques were characterized as FDNA from water samples from Tonegawa river basin in Japan[23]. Livestock facilities near the catchment area apparently had no influence on FDNA prevalence. However, the presence of both FDNA and FRNA in surface

waters from agricultural watersheds was linked to fecal inputs from a nearby hobby farm[14]. Some of the elevated FDNA numbers in our study may have been due to the simultaneous detection of somatic coliphages and FDNA by the host C3000.[24] Based on their ubiquitous nature in surface waters as indicated in this and previous[9] studies it can be concluded that FDNA are not useful as source-specific fecal indicators.

FRNA were detected from water samples collected from most sampling locations and with high abundance in two locations known to be impacted by human activities. These results are in agreement with prevalence of FRNA in waters from Kofu[16] and Tonegawa[23] river basins and those river waters were considered contaminated with human feces. However, others[15, 17, 23, 25-28] reported variable results of 4 to 100%.for FRNA prevalence in samples from different environmental sources.

FRNA were further characterized as belonging to one of four genogroups using separate SYBR green assays. We hoped this method would be less stringent in specificity and be able to characterize a greater proportion of FRNA from environmental samples than in the previous studies.[22, 23] Nevertheless, the method we developed is as sensitive as the probe-based assays[10, 20] and doesn't have any cross-reactivity between the four reference strains used.

Table 3 MSC characterized from waters sampled at six different sampling locations. Phage data was pooled from 14 different sampling intervals for each location

Location	Total MSC, PFU/ml[a]	FRNA, PFU/ml (% total)					FDNA, PFU/ml (% total)
		Animal origin		Human origin		Unclassified	
		GI (MS2[b])	GIV (SP[b])	GII (GA[b])	GIII (QB[b])		
ESPESP	43	1 (2)	0	1 (2)	4 (9)	0	37 (86)
RECVIC	96	1 (1)	8 (8)	4 (4)	13 (14)	0	70 (73)
GABVET	13	0	0	1 (8)	1 (8)	0	11 (84)
ALICAR	82	4 (5)	1 (1)	7 (9)	28 (34)	5 (6)	37 (45)
GFBELM	1	0	0	0	0	0	1 (100)
SABSAL	9	0	0	1 (11)	1 (11)	0	7 (78)
Total	**244**	**6 (2)**	**9 (4)**	**14 (6)**	**47 (19)**	**5 (2)**	**163 (67)**

[a] Denotes total MSC recovered from both water and swab samples from the same location.
[b] Reference FRNA used in RT-PCR assays

Table 4 MSC characterized from water samples collected at various intervals. Phage data pooled from all sampling locations at each interval

Date of sampling	Total MSC, PFU/ml[a]	FRNA, PFU/ml (% total)					FDNA, PFU/ml(% total)	Rainfall[b], cm
		Animal origin		Human origin				
		GI (MS2)	GIV (SP)	GII (GA)	GIII (QB)	Unclassified		
12/19/12	4	0	0	0	1 (25)	0	3 (75)	1.45
01/02/13	15	1 (7)	0	5 (33)	9 (60)	0	0 (0)	5.21
01/15/13	15	0	2 (13)	3 (20)	6 (40)	0	4 (27)	2.41
02/13/13	6	0	0	1 (17)	2 (33)	0	3 (50)	0.51
02/21/13	13	3 (23)	0	1 (8)	1 (8)	0	8 (61)	0.91
03/07/13	14	0	3 (21)	2 (14)	2 (14)	0	7 (50)	0.74
03/20/13	37	1 (3)	0	0	19 (51)	5 (14)	12 (32)	0.94
04/04/13	20	0	0	1 (5)	0	0	19 (95)	0.58
04/17/13	10	1 (10)	0	0	0	0	9 (90)	0.51
05/02/13	17	0	0	0	0	0	17 (100)	0.0
05/14/13	6	0	0	0	0	0	6 (100)	0.0
05/29/13	25	0	4 (16)	0	0	0	21 (84)	0.03
06/26/13	36	0	0	1 (3)	0	0	35 (97)	0.10
08/07/13	26	0	0	0	7 (27)	0	19 (73)	0.03
Total	**244**	**6 (2)**	**9 (4)**	**14 (6)**	**47 (19)**	**5 (2)**	**163 (67)**	

[a] Denotes total MSC recovered from both water and swab samples from the same location.

[b] Two weeks of cumulative rainfall up to the day of sampling. Rainfall data obtained from a weather station located near KSNS Salinas Municipal Airport, Salinas, CA. www.weatherunderground.com/history . GPS co-ordinates: 36.6627865N -121.6063664W.

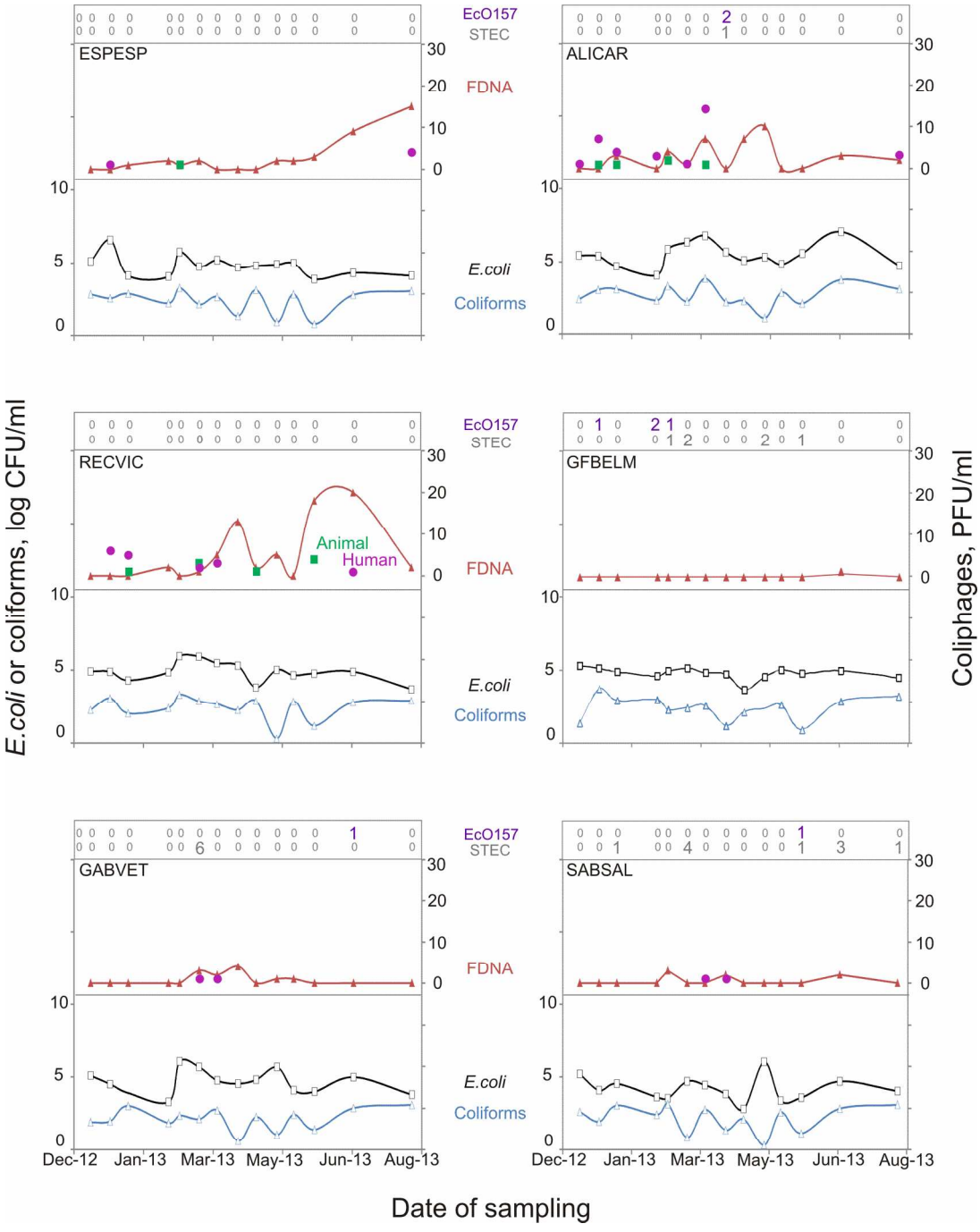

Fig. 2 Seasonal fluctuations in populations of *E. coli*, coliform bacteria, FDNA and FRNA, and incidence of EcO157 and STEC *E. coli*. Numbers for FDNA (filled triangle, red) and FRNA were pooled from water and swabs at each sampling interval. Numbers of FRNA from genogroups II and III in water and swab samples were pooled to represent MSC from human sources (filled circle, purple). Similarly, FRNA of animal (filled square, green) origin were obtained by pooling numbers from genogroups I and IV. Incidence of EcO157 (violet numbers) and STEC *E. coli* (grey numbers) or absence (0) is indicated in the top section of each panel. Seasonal fluctuations for *E. coli* (unfilled square, black) are from swabs and the coliforms (unfilled triangle, blue) are from water samples collected at the same location.

Only 31% of MSC were characterized as FRNA from the four known genogroups. These results are comparatively similar in proportion as reported previously for samples of human sewage or animal waste[29-31] but proportions > 80% were reported for river waters.[15, 16, 28] Thus, the prevalence of FRNA in environmental samples may vary widely depending upon the

source.

Ninety-one percent of all characterized FRNA were from human sources and 90% of them were from locations (RECVIC, GABVET and ALICAR) known to be impacted by human activities (Table 3). Similarly, human-impacted municipal waters contained a high proportion of FRNA originated from humans.[9] A possible explanation for the preponderance of FRNA from humans was their apparent persistence in the environment.[9, 32] However, this is known for members of genogroup II but is not known if QB-like phages (GIII), predominant in this study, were also persistent. Similar to this study, a higher prevalence of members of group III followed by group II in raw sewage was reported in a previous study.[16] Even more intriguing is the detection of 93% (14/15) of FRNA from animal sources from the same three locations impacted by human activity. Both groups I and IV were represented from these samples with group IV prevalent at location RECVIC and group I more prevalent at ALICAR (Table 4). Although, groups I and IV were predominantly of animal origin they were also characterized from municipal waters.[33, 34] Furthermore, these sites are downstream from animal raising operations and are influenced also by the activities of domestic or feral animals. Thus, human activities significantly impact the prevalence and types of FRNA.

Rainfall prior to samplings correlated with the prevalence of FRNA originated from humans at RECVIC, a site known to be impacted by human activities and a similar relationship was observed between recovery of FRNA and rainfall in a previous study.[35] Similar to the current study, sites impacted by urban activity yielded more FRNA during rain events than the agricultural sites.[35]

Nearly eighty percent of all characterized FRNA were isolated during the months of January to March 2013, indicating a seasonality of prevalence. Cooler prevailing monthly mean temperatures ($11.5^{o} \pm 0.9^{o}$C) and high rainfall (7.5 ± 0.6 cm) in Salinas Valley region (www.weatherunderground.com/history) could be responsible for the high occurrence. Similarly, higher incidence of MSC were observed during the months of autumn followed by winter of 2000-2002 in Massachusetts bay[36]. In contrast, FDNA were found to be more prevalent during the warmer months (Table 4) as was similarly observed by Cole *et al.*[9] for FDNA in surface waters potentially impacted by waste discharges or run-offs from land uses. Furthermore, FDNA are known to be more resistant to sunlight[37] compared to FRNA and FRNA are inactivated also at warm temperatures[9] during summer months. Thus, there appears to be a significant relationship between seasonality and the incidence of FRNA and FDNA in surface waters.

Owing to their scarcity, the prevalence of STEC *E. coli* and EcO157 could not be correlated with FDNA or FRNA in this study. The low detection of STEC *E. coli* and EcO157 (Table S1) is not surprising as Wilkes *et al* [14] failed to find any EcO157 during a 7-year period from surface waters from agricultural watersheds. Furthermore, these bacteria were detected predominately in waters from agricultural locations and not from human-impacted sites (Fig. 2). STEC *E. coli* and EcO157 prevalence could not be related also to the seasonal fluctuations in generic *E. coli* or fecal coliforms. Thus, *E. coli* were found to be an unreliable indicator as surrogates for predicting the prevalence of EcO157 or other food and waterborne pathogens.[3, 38]

Since coliform bacteria and *E. coli* fluctuated similarly in waters from agricultural or human-impacted sites, they don't appear to be source specific and could not be used as indicators for source tracking. However, the detection of FRNA from human sources in samples from majority of human-impacted sites and detection during winter months suggest that FRNA are better predictors of fecal pollution of surface waters. Based on these results, that need validation with long term studies, we conclude that both FRNA and coliform bacteria are not predictors of prevalence of STEC *E. coli* in the environment. However, the data on FRNA is invaluable in predicting fecal contamination of waters used for produce cultivation.

Conclusions

FDNA were more prevalent compared to FRNA in surface waters and were found predominantly during summer months, whereas FRNA were prevalent during winter months. Most of the characterized FRNA were sourced to humans and were from human-impacted locations. Of the FRNA sourced to humans, members of genogroup III dominated. STEC *E. coli* and EcO157 were detected mostly from locations impacted by agricultural activities and could not be correlated with the prevalence of MSC. Similar studies, when expanded to include additional produce production regions, areas influenced by animal raising operations and human-impacted sites should provide valuable data on fecal contamination sources that aid in developing methods to control or minimize the spread of EcO157 and other enteric pathogens.

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Notes and references

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† Electronic Supplementary Information (ESI) available: Table S1. Characterization of *E. coli* O157:H7 and Shiga-toxigenic *E. coli* isolated from swab samples collected from various locations. See DOI: 10.1039/b000000x/

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