INTRODUCTION

Prokaryotes are essential components of the marine ecosystem. They account for the bulk of the biomass and have essential roles in various biogeochemical processes such as carbon, sulfur and nitrogen cycling (Azam et al. 1983, Fenchel 1988). Determining the specific prokaryotic composition of the marine microbial community has proven to be difficult due to the inability of many of these microorganisms to be cultured. Fortunately with the advent of advanced molecular techniques, such as those, which utilize ribosomal RNA gene sequences, the detection, identification and enumeration of individual microbial cells without cultivation, is now possible (Amann et al. 1995).

Historically, microbial identification required isolation of pure culture followed by testing for physiological and biochemical traits. Culturable counting techniques such as plate count or most-probable-number (MPN) techniques have been used for quantification of active microbes in environmental samples (Amann et al. 1995). However, direct microscopic counts exceed culturable cell counts by several orders of magnitude (Amann et al. 1995, Fuhrman et al. 1994). This is due to microorganisms that currently are nonculturable as well as culturable cells that exist in a viable-but-non-culturable state (Whitesides and Oliver 1996). The cultured species of the domains of *Bacteria* (eubacteria) and *Archaea* (archaebacteria) are known to represent only a small fraction of the actual prokaryotic community (Ferguson et al. 1984, Jannasch and Jones 1959). The domain *Bacteria* is a highly diverse collection of prokaryotes with numerous culturable species. Whereas the domain *Archaea* is composed of the kingdom *Crenarchaeota*, whose cultured members include extreme thermophiles, and the kingdom *Euryarchaeota*, whose cultured members include methanogens, the extreme halophiles,

and sulfur reducing thermophiles (Prescott et al. 1999). Recently, a third kingdom, the *Korarchaeota*, has been proposed based on sequences collected from hot spring environments and are yet to be cultured (Barns et al. 1996, Pace 1997).

The analysis of gene sequences of the small subunit of ribosomal RNA as phylogenetic markers has helped characterize the marine microbial community (Amann et al. 1995, Schmidt et al. 1991). By using PCR amplification, retrieved sequences can be compared and the identity of prokaryotes in natural samples can be determined, regardless of culturablity. One of the first uses of this molecular technique was the analysis of the composition of marine picoplankton (planktonic organisms with an average diameter of 0.2-2.0μm). Studies revealed that most marine prokaryotes were undescribed species that had not been cultivated (Britschgi and Giovannoni 1991, DeLong 1992, Fuhrman et al.1992, Mullins et al. 1995). Recent phylogenic studies have shown that uncultured crenarchaeotes (referred to as group I archaea) have been discovered in marine plankton (DeLong 1992, DeLong et al. 1994, Fuhrman and Davis 1993, Fuhrman and Davis 1997, Massana et al. 1997), marine and freshwater sediments (Kato et al. 1997, MacGregor et al. 1997, McInerney et al. 1995, Ravenschlag et al. 2000), freshwater plankton (Jurgens et al. 2000), deep sea hydrothermal vent fluids (Huber et al. 2002), and marine animals (McInerney et al. 1995). Uncultured euryarchaeotes referred to as group II archaea have been found in marine plankton (DeLong 1992, DeLong et al. 1994, Fuhrman and Davis 1993,1997, Massana et al. 1997), freshwater plankton (Jurgens et al. 2000), marine sediments (Moyer et al. 1998), deep sea hydrothermal vent fluids (Huber et al. 2002), and the digestive tracts of fish (Van der Maarel et al. 1998). Marine archaea are believed to be abundant and could account for up

to 30% of the total picoplankton in both polar and temperate coastal waters and up to 60% in both mid and pelagic waters (DeLong et al. 1994, Fuhrman and Davis 1997, Massana et al. 1997, 2000).

While marine microbial diversity can be studied by PCR-based phylogenic analysis, the actual community structure cannot be deduced due to potential bias from amplification of certain templates, potential sample contamination, variability in cloning efficiencies for different rRNA gene fragments, and selective priming for certain sequences (Polz & Cavanaugh 1998). By using quantification methods such as fluorescence *in situ* hybridization (FISH), the identity and abundance of the natural community can be determined (Amann et al. 1995).

Fluorescence *in situ* hybridization is a molecular technique that uses fluorescent probes that specifically target rRNA gene sequences of whole cells *in situ,* which allows for the identification and enumeration of nonculturable microorganisms in their natural environment (Amann et al. 1995, Delong et al. 1999). In this technique fluorescently labeled rRNA-targeted oligonucleotide probes penetrate the microbial cell wall and bind to the complimentary targeted rRNA sequence and are then detected by epifluorescence microscopy (Amann et al. 1989, DeLong et al. 1989).

The distribution, quantification and phylogenetic characters of the marine microbial community have been characterized in deep-sea waters using rRNA molecular analysis (Amann et al. 1995, Britschgi and Giovannoni 1991, DeLong 1992, DeLong et al. 1999, Massana et al. 1996, Massana et al. 1999, Murray et al. 1998, Ward et al. 1992), yet there are few reports of these techniques being applied to the estuarine environment. One report of the archaeaplankton of the Columbia River estuary has indicated that

archaea are present in the estuarine environment yet do not form a native estuarine community. This is in contrast with the bacterial community, which seems to form a native estuarine community (Crump and Baross 2000).

Estuaries are characterized as that portion of the earth's coastal zone where there is interaction of ocean water, fresh water, land, and atmosphere. The species composition of the estuarine community is a function of various environmental factors including salinity, turbidity, nutrients, turbulence, and depth. Estuaries tend to have high levels of nutrients many of which are recycled through the activities of microorganisms, and thus are critical in controlling the function and structure of estuarine ecosystems (Day et al. 1989). Because estuaries act as a natural sink for nutrients it is essential to identify those microorganisms that are responsible for the nutrient recycling.

The Cape Fear River estuary drains the largest and most industrialized watershed in North Carolina, and is home to 27% of the state's population (Mallin et al. 2000). Freshwater from the Cape Fear River flows into the Cape Fear estuary, which is characterized as having a salinity gradient from fresh to salt water and is subject to a tidal influence from the Atlantic Ocean. Like many estuaries, it functions as a nursery for marine animals, a habitat for numerous plants and animals species, a storage basin for nutrients and as a buffer zone from storm impact. The estuarine environment is highly susceptible to chemical runoff and other forms of air and water pollution (Day et al. 1989). Therefore to further understand this dynamic environment, characterization of estuarine microorganisms is needed.

The objectives of this study were to quantify the microorganisms of the small eukaryotic (5.0-0.22μm), bacterial and archaeal community of the Cape Fear River

estuary, by using fluorescence *in situ* hybridization (FISH). The archaeal community was further quantified by group-specific analysis. By using FISH the spatial and temporal composition of the planktonic microbial community of the Cape Fear River estuary was analyzed. This study represents the first attempt to detect and quantify marine Archaea in the Cape Fear River estuary.

MATERIALS AND METHODS

Site Description

The Cape Fear River estuary is a coastal plain estuary joined by three major tributaries, The Cape Fear, the Black and the Northeast Cape Fear rivers, in southeastern North Carolina (Figure 1). The system drains numerous swamp forests, which add organic color and acidic tannins to the mainstream. The estuary is influenced by a diurnal tide, which extends upstream northeast from the Atlantic Ocean. It is the most heavily industrialized watershed within North Carolina with approximately 1.5 million people living within its basin (Mallin et al. 2002).

Sample collection, processing, and storage

Water samples were collected monthly during out-going tides at surface and depth (mean 11m) for one year, at three sites along the Cape Fear River estuary. Samples were taken in conjunction with the Lower Cape Fear River Monitoring Project. Numbered sites (Figure 1) were as follows: (1) Channel Marker 18, located near the mouth of the Cape Fear River estuary with a marine salinity range; (2) Channel Marker 42, located near Keg Island, mid-estuary with a mesohaline salinity range; and (3) Navassa, located

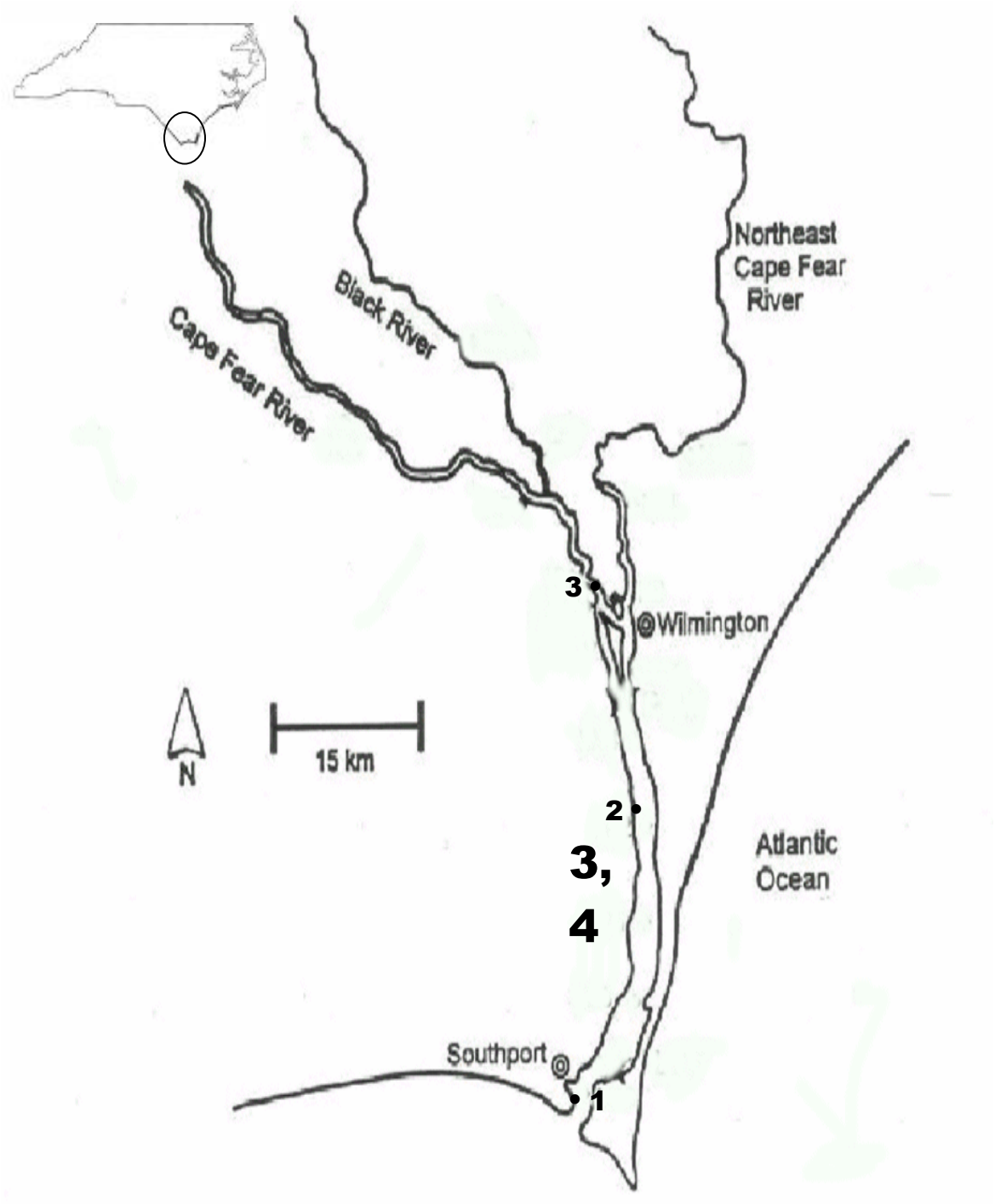

Figure 1. Map of sites sampled from September 2001 to August 2002.

near the head of the estuary, with a oligohaline salinity range. Samples were collected in sterile glass bottles and stored on ice until processed in the laboratory (within 6 hours). Physical parameters were measured with each sample using a Yellow Springs Instrument 6920 (YSI; Yellow Springs, Ohio) Multiparameter Water Quality Probes and stored on a YSI 610D datalogger. Physical parameters included water temperature, salinity, pH, turbidity and dissolved oxygen.

Standard Plate Count

Standard plate count was performed to compare viable versus total (culturable and nonculturable) direct cell counts. Standard spread plate technique, with a nonselective marine medium, salt-water yeast extract (SWYE) agar [1% proteose peptone, 0.3% yeast extract, 2% agar and 1 liter of three salts solution (0.4M NaCl, 0.028M $MgSO_4 \cdot 7\ H_2O$, and 0.01M KCL) pH 7.2-7.4] was used. Plates were done in triplicates, incubated overnight at 37 ºC then counted.

Fluorescence *in situ* hybridization

Samples for FISH assay were fixed overnight at 4° C with 4.0% paraformaldehyde (w/v) in 200mM phosphate buffered saline (PBS, pH 7.2). Following fixation, 1 or 10 ml samples were prefiltered through a 5.0 µm-pore-size type WCN filter (Whatman; Clifton, NJ) and then vacuum-collected onto a 0.2 µm-pore-size polycarbonate GTBP filter (Millipore; Bedford, MA) under a vacuum. Filters were washed once with 0.01M PBS then washed again with nanopure water. Next filtered samples were dehydrated in 50%, then 70%, then 95%, and finally 99% ETOH (3 minutes each) and then dried under vacuum. Finally filters were airdried and stored at –20 ºC until processed.

Fluorescence *in situ* hybridization was performed on the inverted lids of 12-well polystyrene culture dishes (Becton Dickinson and Co.; Franklin Lakes, NJ). Filters were sectioned into eight parts with a sterile razorblade and labeled. Individual sections were placed face up on the inverted lid and 19 µl of preheated (46°C) hybridization solution [0.9 M NaCl, 20mM Tris-HCL (pH 7.4), 35% formamide, and 0.01% SDS] and 1µl of Cy3 -labeled oligonucleotide (50 ng/µl) was added to each section (Amann et al.1995, Glockner et al.1996). Each section was then covered with a glass coverslip and placed into incubation chambers. Each incubation chamber contained 1M NaCl to maintain humidity. Six Cy3-labeled oligonucleotide probes (Operon Qiagen Co.; Alameda, CA) were used for hybridization. These included a universal bacterial probe (EUB338), a universal eukaryotic probe (EUK1379), a universal archaeal probe (ARCH915), the archaeal kingdom Euryarchaeal probe (EURY499), the archaeal kingdom Crenarchaetoa probe (CREN498), and a negative control non-probe (NON338) (Table 1). Incubation chambers then were placed into an incubator at 46° C and incubated overnight. After overnight incubation, the filters were removed and placed face-up and free-floating, in prewarmed (48° C) washing buffer [70 mM NaCl, 20 mM Tris-HCL (pH 7.4), 5 mM EDTA, and 0.01% SDS] for 15 minutes at 48° C. The washing buffer was then poured off and sections were airdried. Once dried, sections were overlaid with 25µl of DAPI (4', 6-diamidino-2-phenylidole) solution (4µg/µl). Sections were incubated in the dark at room temperature for 5 minutes then washed in 50 ml of nanopure water and airdried. Once dried, sections were placed on a glass slide using small drops of Type DF immersion oil (Cargille Laboratories Inc.; Cedar Grove, NJ) under and above the filter section and covered with a glass coverslip for observation.

Table 1. Oligonucleotide probes used in this study.

Probe	Specificity	Sequence (5'-3')	Target[a] site (rRNA positions)	Refs.
ARCH915	*Archaea*	GTG CTC CCC CGC CAA TTC CT	16S (915-935)	43
EUB338	*Bacteria*	GCT GCC TCC CGT AGG AGT	16S (338-355)	1
CREN499	*Crenarchaeota*	CCA GRC TTG CCC CCC GCT	16S (499-515)	7
EUK1379	*Eukarya*	TAC AAA GGG CAG GGA	16S (1379-1394)	1
EURY498	*Euryarchaeota*	CTT GCC CRG CCC TT	16S (498-511)	7
NON338	Negative Control	ACT CCT ACG GGA GGC AGC	16S (338-355)	45

[a] *Escherichia coli* numbering.

Direct Count

Prokaryotic cell densities were estimated by DAPI staining and direct count using epifluorescence microscopy (Porter and Feig 1980). For both FISH and DAPI, filter sections were viewed immediately with an Olympus BH-2 microscope equipped with an HBO 100-W mercury lamp and a 100x, Fluor-APO objective. Appropriate filter sets for DAPI and Cy3 were used (365/395 excitation, 420 emission and 545/565 excitation and 610 emission, respectively). Images were captured using a SPOT RT color camera (Diagnostic Instruments, Inc.) and a computer. Image analysis and cell quantification was performed using Image Pro Plus version 4.1.0.0 for Windows 95/NT/98 (Media Cybernetics, L.P.).

To quantify probe-positive cells, mean values were calculated from 10 random fields for each filter section. Counting results were adjusted by including a negative control probe (NON338) for each section and used to calculate the final probe-positive cell concentration. The fraction of probe-positive cells was calculated as the ratio of the number of probe-positive to the total number of DAPI stained cells after background subtraction of the NON-probe counts for each probe treatment. The cell densities were calculated by multiplying the fraction of probe-positive cells by the total prokaryotic cell concentration estimated from direct epifluorescence microscopic counts (Porter and Feig 1980, DeLong et al. 1999).

Data Analysis

Total viable cell counts were calculated from the number of colony forming units (CFU) growing on SWYE (0.1 ml). Total viable cell count (TVCC) then was calculated

as a fraction of total direct cell count (TDCC) by DAPI for percent viable of total cell count.

The effect of site, depth, and time on microbial densities was tested using Analysis of Variance (Two-way ANOVA) using JUMPIN software Version 3.2.1 (SAS Institute Inc.). The mean of each probe-positive section was compared to each site at surface and depth for the twelve months sampled. Two-way ANOVA also was used to determine the effect of physical parameters on microbial densities.

RESULTS

Total Cell Count

Concentrations of the total microbial community enumerated by DAPI staining and epifluorescence microscopy ranged from 3.4 to 52.2 x 10^4 cells ml^{-1} over the 12-month sampling period. Whereas the viable microbial community enumerated by standard spread plate ranged from 0.3 to 16.9 x 10^3 cells ml^{-1}. Total viable cell counts accounted for 1-6% of the total direct cell count as detected by DAPI. TVCC counts ranged from a mean low of 1.4 x 10^3 ml^{-1} in February and April to a mean max of 8.1 x 10^3 ml^{-1} in July. TDCC was higher with a mean low of 5.7 x 10^4 ml^{-1} in January and a mean high of 3.8 x 10^5 ml^{-1} in July (Table 2). TDCC showed seasonality with a decrease of cell counts in the colder months and an increase of cell counts in the warmer months. TVCC also showed changes with season but had greater variability between months (Figure 2). There was a significant effect of season on both the TVCC and TDCC ($F=4.3184$ and $F=15.2929$, $P<0.0001$ respectively) yet depth was not a significant influence ($P>0.05$) on either count.

Table 2. Mean total viable cell count; mean total direct cell count and percent of culturable microorganisms for all sites.

Month[a]	Total viable cell count ($10^3 ml^{-1}$) (mean ± SD)	Total direct cell count ($10^3 ml^{-1}$) (mean ± SD)	Percent culturable[b] (%)
Sep-01	3.3 ± 1.3	93.2 ± 12.2	5
Oct-01	4.2 ± 2.0	89.7 ± 20.5	5
Nov-01	3.4 ± 1.9	66.7 ± 25.8	5
Dec-01	3.3 ± 4.1	57.3 ± 18.4	6
Jan-02	3.2 ± 3.5	80.7 ± 24.8	4
Feb-02	1.4 ± 0.9	85.9 ± 33.1	2
Mar-02	2.2 ± 0.8	93.4 ± 39.4	3
Apr-02	1.4 ± 0.9	164.5 ± 63.9	1
May-02	5.1 ± 2.3	251.5 ± 145.5	3
Jun-02	7.3 ± 4.1	216.2 ± 59.5	4
Jul-02	8.1 ± 4.6	378.0 ± 15.5	3
Aug-02	3.7 ± 1.3	343.1 ± 75.2	1

[a] Sampled at surface only September 2001.
[b] Percent difference calculated as TVCC compared to TDCC.

Table 3. Mean total viable cell count; mean total direct cell count and percent of culturable microorganisms for sites sampled at surface and depth from September 2001 to August 2002.

Site[a]	Total viable cell count ($10^3 ml^{-1}$) (mean ± SD	Total direct cell counts ($10^3 ml^{-1}$) (mean ± SD)	Percent culturable[b] (%)
1 surface	2.1 ± 1.2	135.5 ± 70.7	2
1 depth	4.2 ± 4.7	152.7 ± 110.9	3
2 surface	3.6 ± 2.8	128.3 ± 83.6	3
2 depth	3.7 ± 2.5	161.2 ± 148.0	2
3 surface	5.9 ± 3.3	190.9 ± 158.2	3
3 depth	5.6 ± 4.0	215.1 ± 175.3	3

[a] Sampled at surface only September 2001..
[b] Percent calculated as TVCC compared to TDCC.

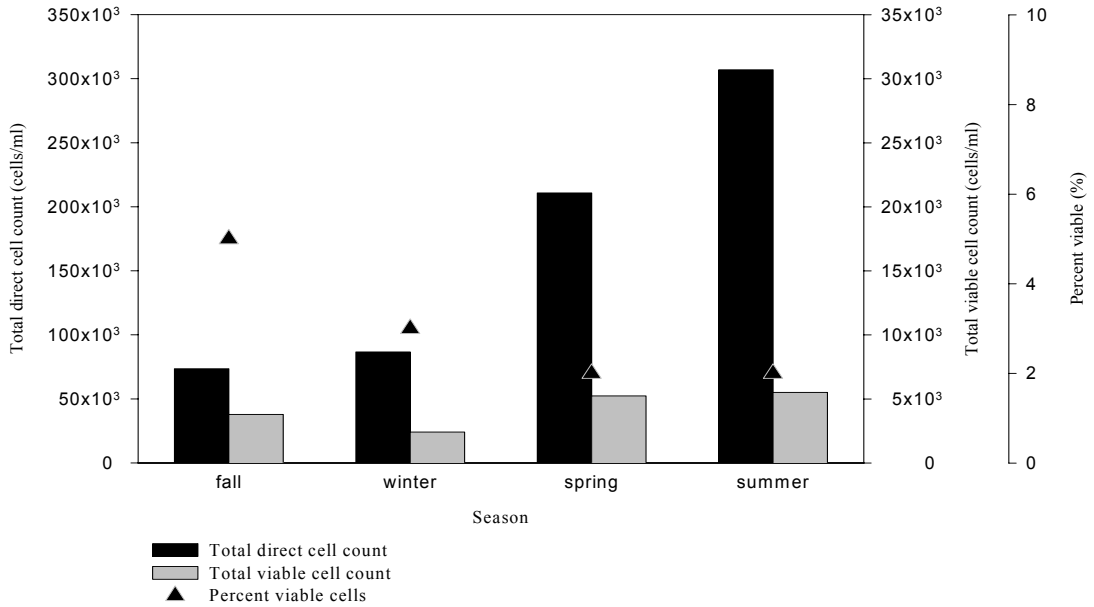

Figure 2. Total direct cell count (cells/ml) compared with total viable cell count (cells/ml) and percent viable (%) of total direct cell count from September 2001 to August 2002. Seasons have been grouped by water temperature: fall; October, November, December, winter; January, February, March, spring; April, May, June, and summer; July, August, and September.

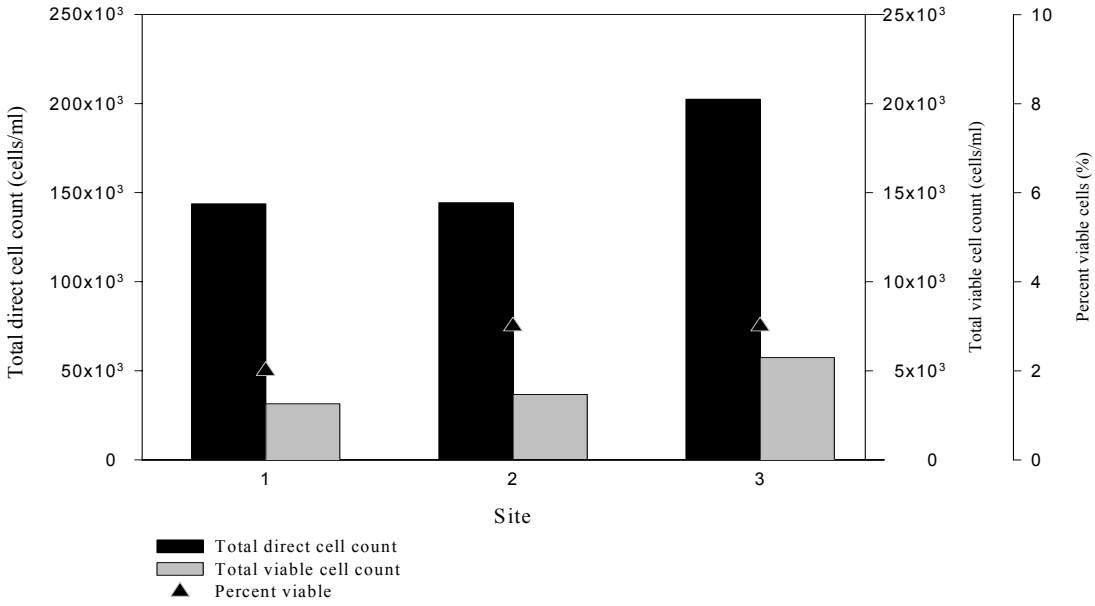

Figure 3. Total direct cell count (cells/ml) compared with total viable cell count (cells/ml) and percent viable (%) of total direct cell count from September 2001 to August 2002. Surface and depth cell counts have been grouped for each site.

Site also had a significant effect on TVCC and TDCC (F=7.5143, P=0.02 and F=5.5058, P=0.0067 respectively). Viable cell count at all sites ranged from mean 2.1 to 5.9 x 10^3 cells ml^{-1}. Direct cell count at all sites ranged from mean 1.3 to 2.2 x 10^5 cells ml^{-1} (Table 3). The nearly freshwater station, site 3, had the greatest total cell count ml^{-1} and was significantly different from both site 1 and 2 ($P<0.05$) (Figure 3). There was no detectable significant influence between site and depth, site and time, depth and time or time, site and depth on TVCC or TDCC ($P>0.05$).

Physical Parameters

For the twelve months sampled, physical parameters including site salinity, monthly water temperature, pH, turbidity, and dissolved oxygen were recorded. Salinity at the sampling sites ranged from a mean low of 6 ppt to a mean high of 34 ppt with the samples collected at depth being slightly more saline than the surface samples (Table 4). Site 1 had the highest salinity (range 21.1 to 37.7 ppt) of the sites sampled. Site 2 was mesohaline and showed the greatest variability (range 6.4 to 30.0 ppt). Site 3, the least saline of the sites (range 0.1 to17.5 ppt) also showed variability over the sampling period. Salinity had a small but significant negative effect on both TDCC and TVCC (F=4.8106, P=0.0321 and F=4.2688, P=0.0430 respectively). Water temperature ranged from a mean low in January of 9.8 ºC to a mean high of 29.4 ºC in July (Table 5). Water temperature caused a small but significant increase on both TDCC and TVCC (F=15.2156, P=0.0002 and F=7.2471, P=0.0091 respectively). Water temperature did not vary greatly between surface and depth or between sites within the same month. The other collected physical parameters including, turbidity, pH, and DO, did not have a significant effect on total cell distribution or quantity.

FISH Assay

Total probe-positive cells ranged from 1.8 to 31.5 x 10^4 cells ml^{-1} during the months sampled between September 2001 to August 2002. Probe-positive cells ranged from 19 to 83% with a mean of 55% of TDCC. Probe-positive cell quantities varied greatly over the 12-month sampling period. During the cold winter months: January, February, and March (mean water temperature 12 ºC), probe-positive cells accounted for 29% (mean) of the total direct cell count. During the summer months: July, August and September (mean water temperature of 28 ºC), probe-positive cells accounted for 79% (mean) of the TDCC (Figure 4).

Changes in season did have a significant effect on probe-positive cell quantities. Eukaryotes were the least affected by changes in season ($F=2.9062$, $P= 0.0101$). They ranged from 0 to 2.3 x 10^5 cells ml^{-1} and accounted for 6% to 27% of the TDCC. Bacteria and Archaea were both highly affected by changes in season ($F=17.4269$, $P<0.0001$ and $F=12.5070$, $P<0.0001$, respectively). Bacteria ranged from 0.36 to 272.3 x 10^3 cells ml^{-1} and accounted for the greatest portion of probe-positive cells as well as 8% to 44% of TDCC. Archaea ranged from 0 to 1.4 x 10^5 cells ml^{-1}. This domain accounted for the smallest portion of hybridized cells and between 2% to 22% of TDCC (Table 4) (Figure 5). Within the Archaeal domain, group I and II probe-positive cells accounted for 21 to 99% of counted cells. Euryarchaeotic (group II) probe-positive cells ranged from 0 to 8.0 x 10^4 cells ml^{-1} and accounted for the majority of archaeal cells and 1 to 11% of the TDCC ($F=8.2637$, $P<0.0001$). Crenarchaeotic (group I) probe-positive cells ranged from 0 to 44.2 x 10^3 cells ml^{-1} and accounted for 0% to 8% of the TDCC ($F=5.7207$,

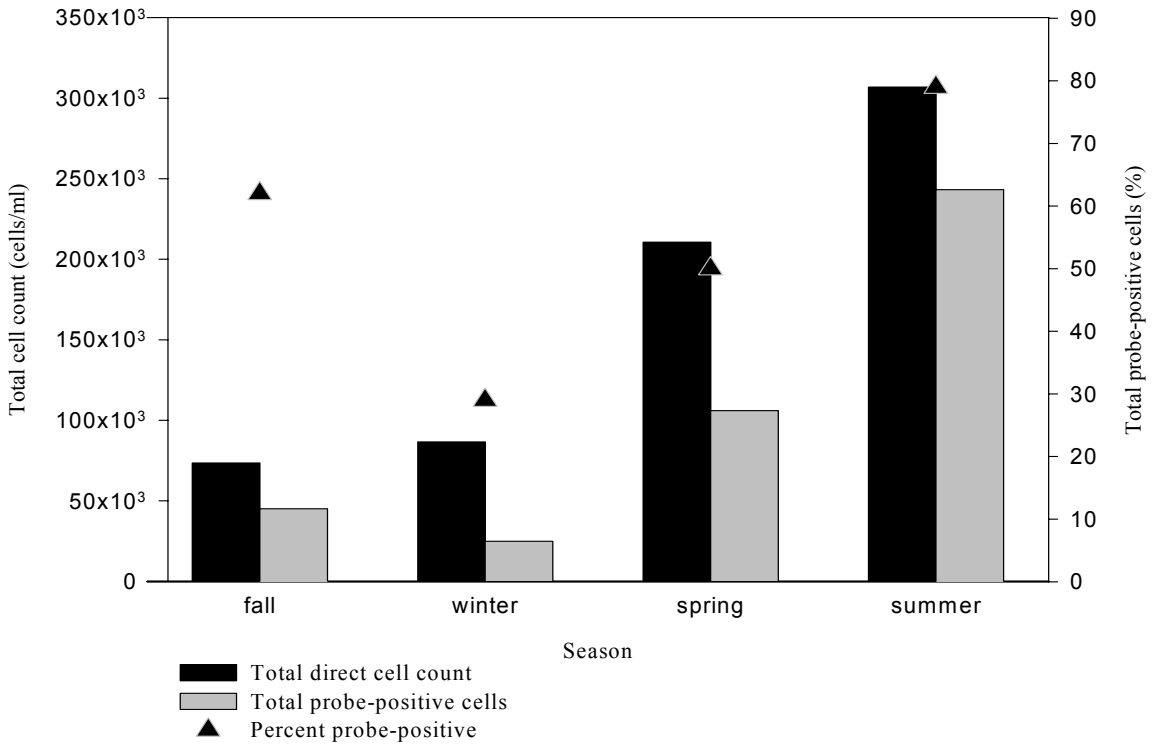

Figure 4. Total direct cell count (cells/ml) and total probe-positive cell count (cells/ml) compared to percent probe-positive cells (%) of total cell count. Counts have been grouped by seasons from September 2001 to August 2002.

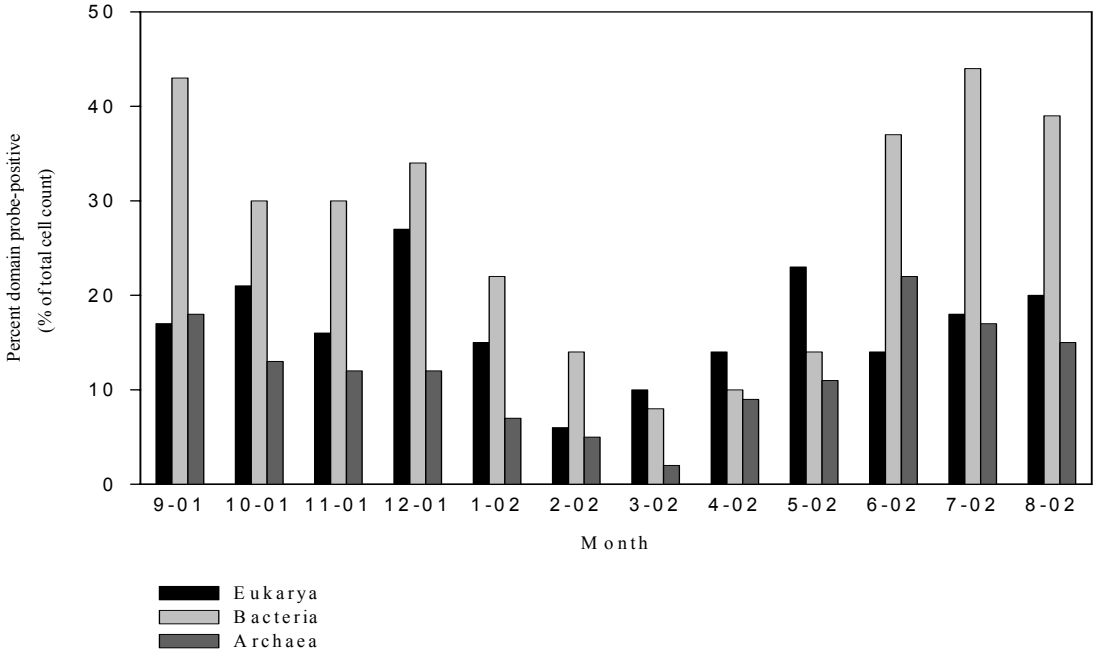

Figure 5. Percent probe-positive cells (%) of the domains; Eukarya, Bacteria, and Archaea, of the total cell count from September 2001 to August 2002.

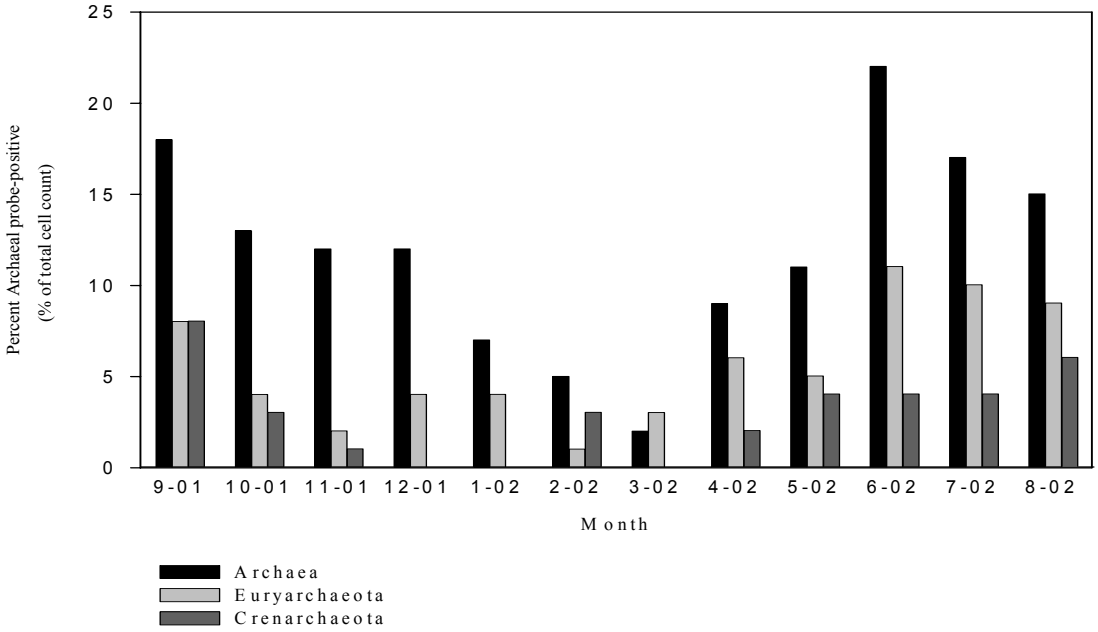

Figure 6. Percent Archaeal probe-positive cells (%) of the domain Archaea, and the subdomains, Euryarchaeota and Crenarchaeota, of the total cell count from September 2001 to August 2002.

P<0.0001) (Table 5) (Figure 6). Site did not have a significant effect on probe-positive densities (P>0.05). However, Crenarchaeota counts were slightly affected by site salinity (F=3.1230, P=0.0573) with an increase in cell density as site salinity decreased.

Warmer water temperature caused a significant increase in the number of Bacteria and Archaea (F=11.7335, P=0.0011 and F=9.6698, P=0.0029 respectively) however Eukaryotic numbers were not significantly affected (P>0.05). As water temperature decreased, the proportion of probe-positive microorganisms decreased to a low of approximately 20% of the TDCC. As water temperature increased, probe-positive densities increased accounting for approximately 80% of the TDCC. Members of the domain Bacteria were the most common microorganisms, accounting up to 40% of the microbial community within the summer months and 14% within the winter months. Archaea followed a similar pattern but made up a smaller fraction of the microbial community, approximately 17% in the summer to only 5% in the winter. Both Bacteria and Archaea showed a winter low in the month of March followed by a summer bloom beginning in June and decreasing in October. The Eukaryotic community was not significantly affected by temperature and showed less variation between months, with a high in the summer months of 18% a low of 11% in the winter and a fall maximum of 27%. Unlike the Bacteria and Archaea, the Eukaryotes had a spring bloom beginning in March and dominated the prokaryotic community until the summer bloom of Bacteria and Archaea in June (Figure 7). Other physical parameters including: salinity, pH, turbidity and DO did not have a significant effect on bacterial or archaeal densities. Eukaryal density also was not affected by salinity, pH, or turbidity but was affected by DO (F=6.3151, P=0.0147).

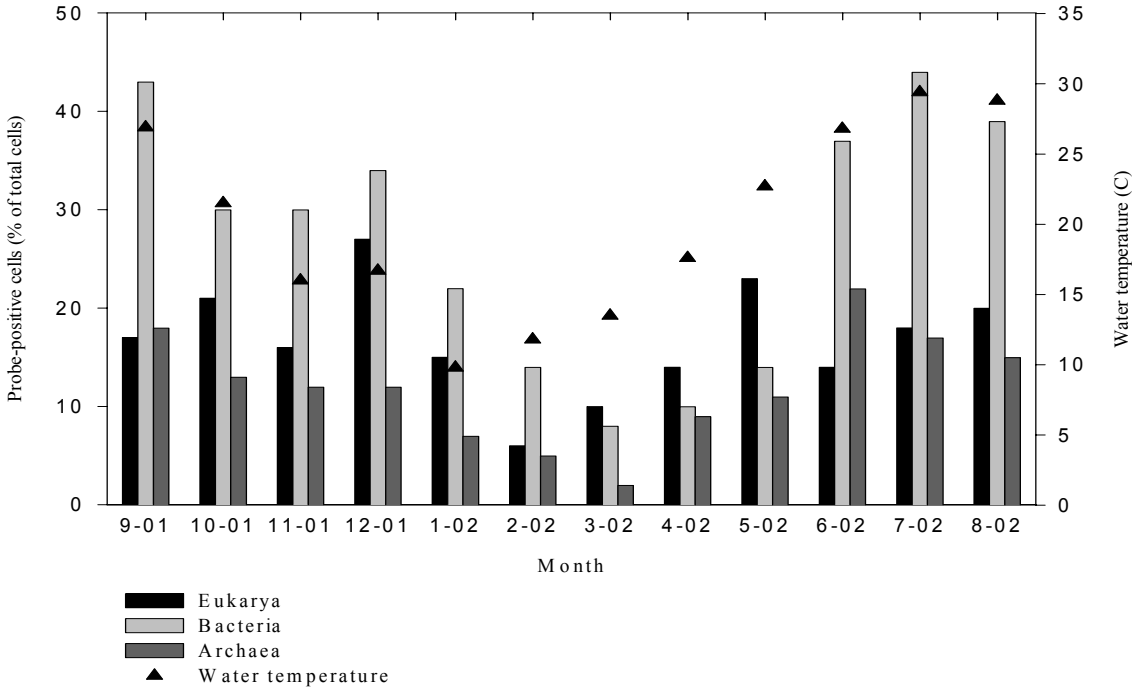

Figure 7. Percent probe-positive cells (%) of the domains; Eukarya, Bacteria, and Archaea, of the total cell count compared to water temperature (C) for the twelve months sampled.

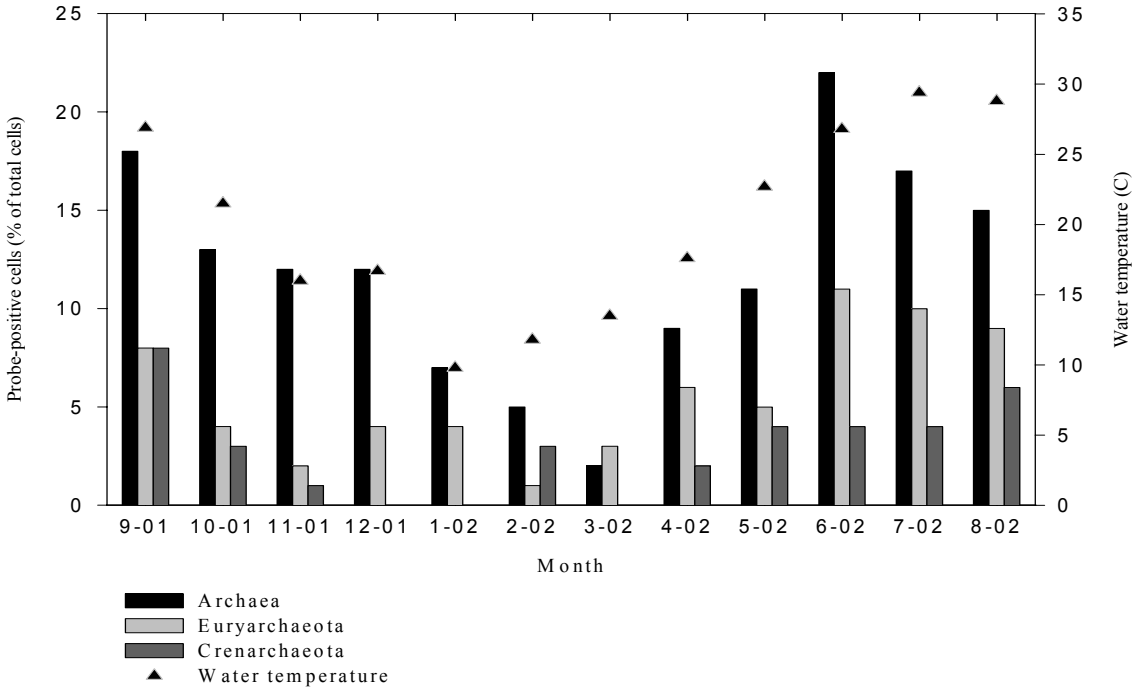

Figure 8. Percent probe-positive cells (%) of the domain Archaea and the subdomains, Euryarchaeota and Crenarchaeota of the total cell count compared to water temperature (C) for the twelve months sampled.

Within the Archaeal domain, water temperature had a significant effect on the Euryarchaeota density only (F=8.5946, P=0.0048). The Euryarchaeota dominated the archaeal population throughout the months sampled except in February where Crenarchaeota were higher (30%) compared to Euryarchaeota (15%). Both kingdoms showed low densities in the winter and a high in the summer months with Crenarchaeota not detected in December, January, and March (Figure 8). Crenarchaeota was not significantly affected by water temperature (P>0.05). It was however slightly affected by salinity (F= 3.9207, P=0.0524) and DO (F=7.2636, P=0.0092).

DISCUSSION

The total viable cell count of the Cape Fear River estuary represented only a small fraction (1-6%) of the total direct cell count of the planktonic microbial community, which is consistent with previous studies regarding culturability (Ferguson et al. 1984, Jannasch and Jones, 1959). As expected, both site and water temperature had an affect on the quantity of cells. There was an increase in cell numbers as cells became more active in the spring and summer months as optimum temperatures were reached. An increase in cell numbers also was seen as salinity decreased indicating that this environment favored further growth. The total microbial abundances were low (3.4 to 52.2 x 10^4 cells ml^{-1}) compared to most coastal temperate environments and could be related to the drought in the region during the sampling period.

While TDCC allows for quantification of total cells within a mixed population, it does not allow for identification and quantification of cell types. To further understand the natural microbial community structure, the molecular technique, FISH was utilized.

Of the total cells stained by DAPI, 55% (mean) of those cells were probe-positive. These percentages fall within expected results when FISH is used in conjunction with the bright fluorochrome Cy3 and image microscopy (Fuhrman and Ouverney 1998). Water temperature had a significant affect on cell-probe hybridization. The percentage of probe-positive cells increased as temperatures increased in the spring and summer months and decreased as water temperature decreased in fall and winter. This coincides with previous studies that suggest probe hybridization intensities decrease as cellular rRNA content decreases in starved or nongrowing organisms which accounts for the low percentages detected in the fall and winter months (Amann et al. 1995).

In the Cape Fear River estuary, planktonic microorganisms of each domain, Bacteria, Eukarya and Archaea, were detected. The temporal distribution of all of the domains was similar in pattern, with an increase in cell numbers in the spring and summer months followed by a decrease in the fall and winter months. Bacteria dominated the estuarine microbial community and accounted for up to 44% of the total microbial population. Eukaryotes accounted for up to 27% of the total microbial population, while Archaea comprised the least amount of the total microbial community (22%). These findings are consistent with previous studies, which suggest that surface planktonic Archaea make up a small yet significant percentage of the total microbial population. It has been suggested that Archaea are possibly outcompeted for resources by other microbial populations in less extreme environmental conditions yet dominate in more extreme environments such as deep pelagic waters or cold Antarctic waters (Murray et al. 1998). In this study the temperate and mesotrophic conditions of the Cape Fear River estuary would be consistent with a mesophilic environment, which could account

for the relatively low abundance of Archaea. In previous studies, archaeal densities were found to increase as depth increased (Furhman and Ouverney 1998, Massana et al. 1997, Pernthaler et al. 2001), yet in this study it was found that depth did not have a significant effect on microbial abundances, suggesting that microbial distribution in this relatively shallow estuary was well mixed.

Members of the kingdom Euryarchaeota (group II archaea) were the dominant archaeal organisms within the Cape Fear River estuary. Euryarchaeota comprised up to 11% of the total microbial population, whereas Creanarchaeota (group I archaea) comprised up to 8% of the total microbial population. Both groups were affected by water temperature (group II significantly only) where an increase in cell numbers was found with increasing temperatures in the spring and summer months and a decrease in the fall and winter months. Crenarchaeota were below detection in the months of December, January and March. It is possible that Crenarchaeota were affected by water temperature, but due to low sample numbers an effect was not seen. This archaeal distribution is consistent with previous studies that have found group II archaea dominate the archaeal population at surface waters and decrease with depth while group I archaea are dominant in subsurface and deep pleagic waters, suggesting zonation between the archaeal kingdoms (Massana et al. 1997 and Karner et al. 2001). Of the total hybridized archaeal cells 1 to 9% were not detected by either group II or I probe (Figure 9). This could be a result of: cells that were present but were not detected by image analysis, ARCH915 oligonucleotide probe bound unspecifically to some bacteria (Pernthaler et al. 2001), or another novel archaeal group was present. Perhaps this unrepresentative group is a native estuarine archaea yet to be discovered.

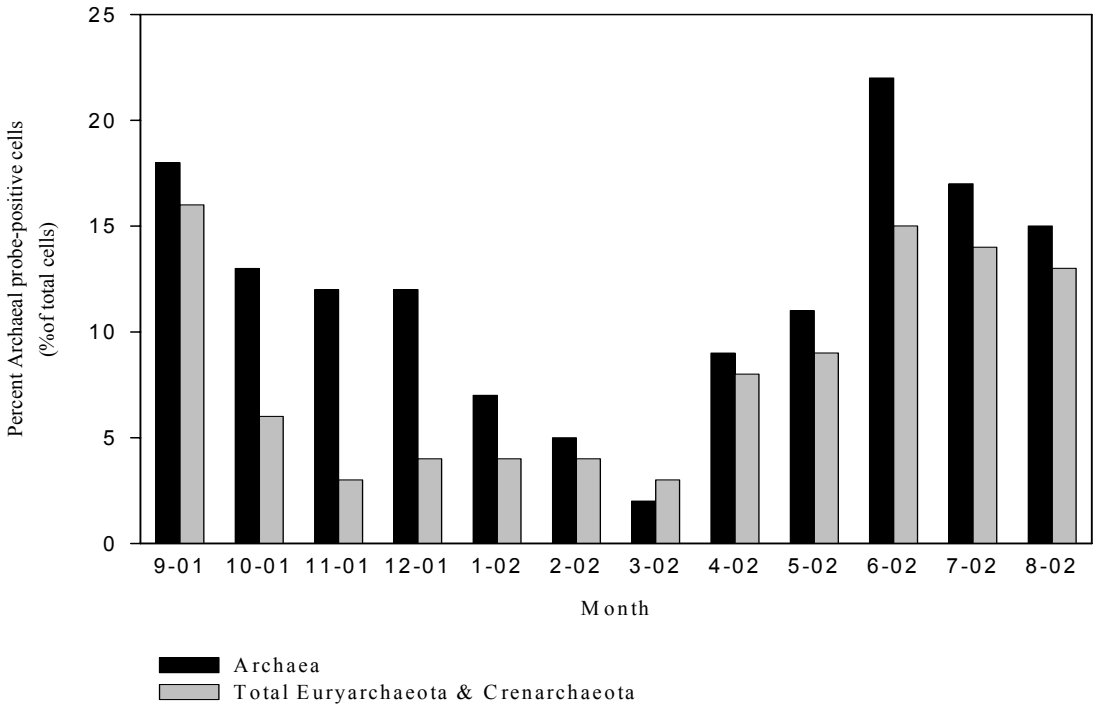

Figure 9. Percent probe-positive cells (%) of the domain Archaea compared to the total percent probe-positive cells of the subdomains, Euryarchaeota and Crenarchaeota, of the total cell count, for the twelve months sampled.

CONCLUSION

This study found that planktonic Archaea comprise a small yet significant portion of the temperate Cape Fear River estuary and their distribution is affected by water temperature. Further analysis must be done to determine if these cells are native estuarine, riverine or coastal archaea. These Archaea are probably aerobic or facultative due to their viability in the water column, while their biogeochemical role in the microbial community is unknown. I believe this is the first report of planktonic Archaea in an Atlantic coastal estuary.