

A Cohort Study to Determine the Epidemiology of Estuary-Associated Syndrome.

Elizabeth Turf¹, Lily Ingsrisawang¹, Megan Turf¹,
Virginia Commonwealth University,
J.D. Ball², Michael Stutts², Eastern Virginia Medical School
John Taylor³, Veterans Affairs Medical Center
Suzanne Jenkins⁴, Virginia Department of Health

INTRODUCTION

Estuary-Associated Syndrome (EAS) is the name given to a potential illness characterized primarily by changes in an individual's cognitive abilities, including acute onset of memory loss or the sudden inability to solve simple problems. Other possible signs of illness include respiratory symptoms, skin rash, or gastrointestinal distress. This illness appears to arise following exposure to toxin produced by *Pfiesteria piscicida*, or other toxic dinoflagellates, that resides in estuary waters.

In 1988, researchers at the College of Veterinary Medicine, North Carolina State University, observed the unusual death of fish in laboratory tanks following exposure to water from the Pamlico River Estuary in North Carolina (Smith et al., 1988). A toxic dinoflagellate was identified in association with the fish deaths and researchers at North Carolina State University were able to reproduce the fish toxicity in a laboratory setting (Burkholder et al., 1992). The organism was named *Pfiesteria piscicida* (Lewitus et al., 1995; and Steidinger et al., 1996). In 1995, this dinoflagellate was found in the Chesapeake Bay (Lewitus et al., 1995) and more

recently in creeks and rivers of Maryland and Virginia (Marshall, personal communication 1999; Grattan et al., 1998). It is now generally recognized that there is a complex of *Pfiesteria*-like dinoflagellates, including *P. piscicida* and an estimated ten or 11 similar organisms. These have been referred to as *Pfiesteria*-complex organisms and more recently as *Pfiesteria*-like organisms (PLOs).

PLOs have a complex life cycle and reside in different forms in the sediment or the water column of estuarine waters. They appear to require live finfish or their secreta for transformation to a toxic phase with subsequent release of a powerful exotoxin(s). Because of the many different forms for these organisms, speciation is difficult and the accepted method depends on scanning electron microscopy (SEM). Screening for PLOs in water samples is currently done using a light microscope with SEM performed if high concentrations of PLOs are seen. Different laboratories have developed molecular methods to identify these organisms in water samples and these methods are being tested (Oldach et al., 1998; Rublee et al., 1999). Work is also ongoing to develop a test to analyze released toxins.

1 Survey and Evaluation Research Laboratory, Virginia Commonwealth University, Richmond, VA
2 Neuropsychology Center, Eastern Virginia Medical school
3 Department of Neurology, Veterans Affairs Medical Center, Richmond, VA
4 Virginia Department of Health

Fish lesions have multiple causes including wounds, toxic chemicals, stress, and infectious agents such as viruses, bacteria, parasites and fungi. Environmental studies have temporally associated a characteristic fish lesion with *Pfiesteria piscicida* in estuarine waters, but it is unknown at this time what role *Pfiesteria* and/or its toxin(s) play. In laboratory studies, *Pfiesteria* toxin(s) causes skin erosion but it is difficult to identify this same damage in a natural setting due to opportunistic bacteria that rapidly invade an eroded area and further degrade the tissue. Since 1984, these characteristic fish lesions have been seen during routine sampling of fish in Virginia waters conducted by the Virginia Institute for Marine Science (VIMS) and the presence of fish with lesions in Virginia waters is one factor used when considering the potential danger to humans of exposure to waters.

The first reported human health effects of *Pfiesteria* were in laboratory workers exposed to toxic cultures of the dinoflagellate. The reported effects identified included epidermal lesions, respiratory problems, gastrointestinal symptoms, disorientation, immunologic compromise, short-term memory loss and/or severe cognitive impairment. Most of these symptoms reversed over time (Burkholder and Glasgow, 1997). In 1995, a review of persons exposed to fish kill events in North Carolina estuaries produced inconclusive results and further epidemiologic study was recommended to determine whether or not a risk was associated with exposure to these *Pfiesteria*-related events (Morris, 1996).

In August and September of 1997, fish kills occurred on several rivers that feed into the Chesapeake Bay including the Pocomoke that runs through Maryland and Virginia. PLOs were identified in water samples collected from each of the affected waters. In September, individuals reporting symptoms of illness with exposure to the Pocomoke River, together with unexposed controls, were examined. Although general physical and neurological examinations were within normal limits, exposed persons were found to have significant differences on tests of learning and higher cognitive function with no known history to explain the findings (Grattan et al., 1998). In addition, several of the individuals had unexplained skin lesions. Repeat testing of those displaying abnormalities showed that the effects appear to be reversible, with scores returning to normal after six months.

In Virginia in the fall of 1997, nine individuals were identified with the requisite exposure and symptoms meeting the suspect case criteria defined at a multi-state workshop in September (CDC, 1997). Five of the nine agreed to be evaluated by the medical team assembled by the Virginia Department of Health (VDH). General physical and neurologic examinations were performed on all and found to be normal, with one person displaying a skin rash. Four of the five patients submitted to a full neuropsychologic battery of tests. Two patients showed mild problems in regards to attention, memory and verbal learning tasks that were unexplained by their histories (Personal communication from Dr. Suzanne Jenkins, VDH). Based on the education and present vocation of these two men, none of these impairments was anticipated. Both were working on boats on the Pocomoke River at the time of the August 1997 fish kill.

In November 1997, researchers and public health officials in North Carolina recruited 33 persons exposed to estuary waters with possible PLO activity and 33 that were unexposed (offshore exposure only) for clinical evaluation. The study found no statistically significant differences between the groups except for visual contrast

sensitivity (VCS) scores, a test not done on persons in Maryland or Virginia in 1997. The estuary watermen were found to have a significantly decreased mean VCS when compared to the offshore group (Hudnell, 1998). Visual contrast sensitivity describes how well a person is able to detect visual patterns. It is considered an indicator of neurological function between the retina and cortex, a pathway that has been found to be very sensitive to neurotoxins. The VCS test is recommended in neurological test batteries as a way to detect subtle neurotoxic effects.

In order to learn more about this possible syndrome and to determine if a causal relationship exists between association to waters containing *Pfiesteria* or other toxic PLOs and illness, cohort studies in Maryland, North Carolina and Virginia were funded by the Centers for Disease Control and Prevention (CDC). In Virginia, CDC funding through VDH supports the study being done by the Survey and Evaluation Research Laboratory (SERL) at Virginia Commonwealth University with assistance from researchers at the Medical College of Virginia/Virginia Commonwealth University, Eastern Virginia Medical School and the University of Virginia. This study is being conducted in collaboration with researchers at Old Dominion University (ODU), the Virginia Institute of Marine Science (VIMS) and the Department of Environmental Quality (DEQ) who are gathering information on the environmental aspects of Virginia's waters. The objectives of the study include:

1. Determine the association between exposure to estuary waters containing PLOs and possible EAS.
2. Characterize the clinical signs and symptoms of EAS.
3. Determine the incidence and prevalence of EAS.
4. Identify risk factors and exposure conditions required for illness.

MATERIALS & METHODS

A cohort design was chosen by all three states. Individuals with varying levels of exposure to estuary waters potentially containing *Pfiesteria* or PLOs were recruited and will be observed for a four to five year period looking for exposure to *Pfiesteria*/PLOs and any subsequent change in health status. The study design calls for baseline medical examinations with follow-up exams twice yearly. All participants are interviewed biweekly to gather information regarding when and which waters they were exposed to, and what type of exposure occurred during the previous two weeks. In addition, environmental data are being collected to look for *Pfiesteria* or PLOs in Virginia waters.

Participants.

The report of health effects in association with the fish kill in the fall of 1997 had a very negative effect on Virginia's seafood industry that year resulting in a substantial loss of revenue. Therefore, when recruitment for this study began there were a lot of negative feelings among Virginia's waterpersons regarding the study. It was believed that a study concentrating on *Pfiesteria* would draw media attention and cause more harm to their livelihood. Due to the negative feelings on the part of potential participants and the time commitment being requested, it was decided that a convenience sample of volunteers was appropriate for this study. Researchers agreed that better cooperation from participants and fewer persons lost to follow-up over a five-year time-frame would greatly improve the validity of the study and make up for the lack

of random selection of participants. Recruitment efforts included presentations to Watermen Association Meetings; distribution of fliers to licensed waterpersons and different seafood-related businesses; and several newspaper articles and radio spots describing the study and requesting volunteers.

All persons who volunteered for the study were initially screened over the phone by interviewers in SERL's Field Operations. Persons were eligible for the study if they:

- Were between 18 and 70 years of age;
- Had no history of neurologic disease such as: stroke, brain tumor, seizures or epilepsy, encephalitis/meningitis, multiple sclerosis, Parkinson's/Alzheimer's disease, dementia;
- Had no history of head injury with loss of consciousness for more than 30 minutes;
- Had no history of treatment for alcohol or drug abuse; or
- Had no condition that made it impossible for them to complete the neuropsychological battery of tests.

During the initial recruitment in 1998, persons with varying number of hours on the water were accepted into the study. Those who reported fewer than eight hours per week on the water were considered to be 'community controls.' Recruitment conducted in early 1999 was limited to individuals who spend at least eight hours per week on the water a minimum of six months of the year.

Exposure Monitoring.

Participants report exposure and health data to SERL every two weeks, either through a telephone interview or via an electronically transmitted form. The information includes specifics regarding water exposure and health. Geographic data are coded using the EAS Study Map that is divided into grids based on the U.S. Geological Survey map of the Chesapeake Bay and its tributaries (Figure 1). The quadrants of the map are labeled with a letter (east and west) and a number (north and south). Participants report which quadrants they were in during the previous two weeks, how much time was spent in each quadrant and whether or not they observed anything unusual. If illness occurred during the same time period, symptoms are recorded. Exposure to PLOs is determined by comparing exposure data obtained from participants to water characteristics identified through environmental monitoring (Everton et al., 1999).

Medical Examinations.

The case definition used for possible EAS is one developed by a working group in the fall of 1997 and published again in May 1999 (CDC, 1999). It is as follows:

- Exposure to estuary waters within 2 weeks of onset of illness, and
- Memory loss or confusion, and/or
- 3 or more of: headache, skin rash/burning, eye irritation, respiratory irritation, gastrointestinal illness or muscle cramps, and
- No other explanation for findings.

Medical exams are conducted four weekends a year in areas where the participants reside: two weekends are spent on the Eastern Shore and two along the western side

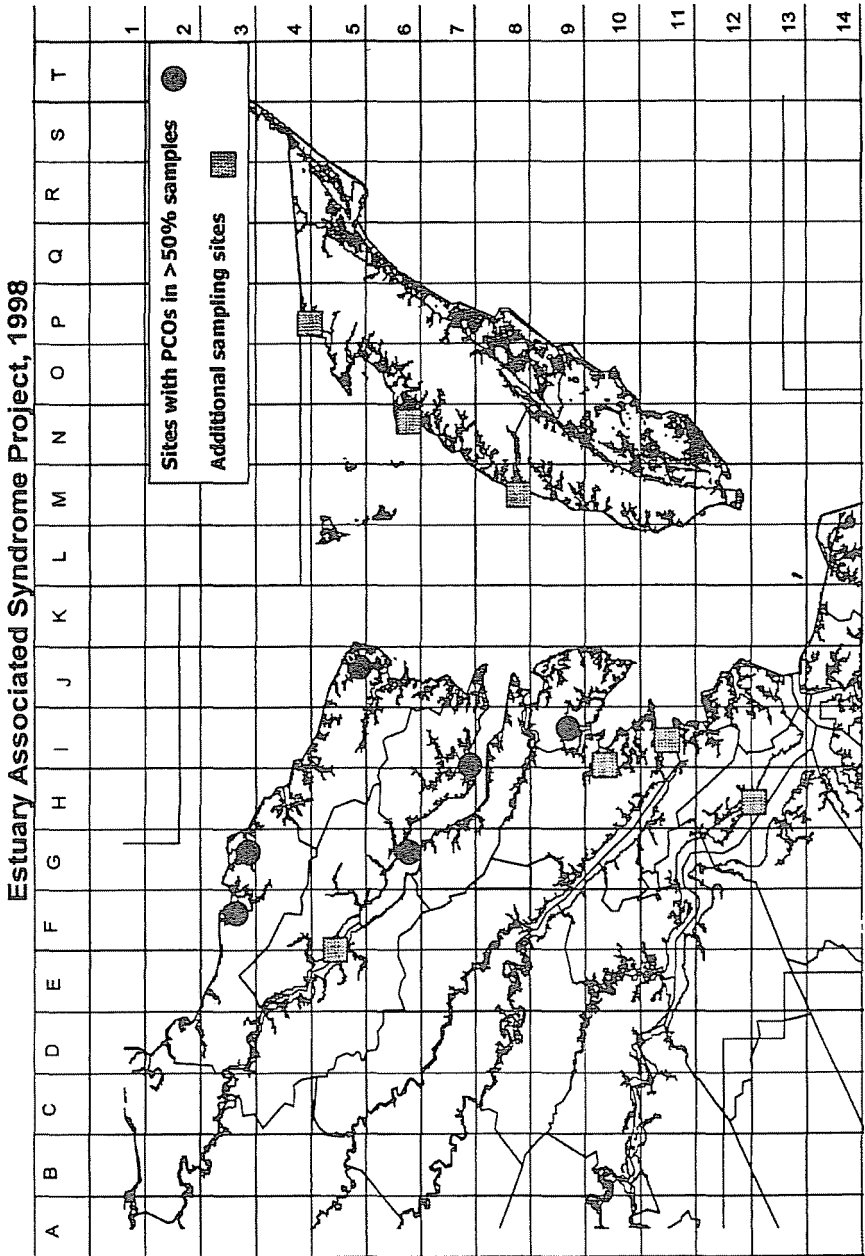


FIGURE 1. Lower Chesapeake Bay sampling sites. Locations in which PLO were found in > 50% of the samples represented by black circles, < 50% of the samples gray squares.

of the Bay. The medical exam includes an examination by an internist, neurologist and dermatologist; a laboratory work-up with a full chemistry panel, complete blood count and urinalysis; vision testing that consists of a Snellen visual acuity test, VCS testing and color vision testing; neurocognitive testing; and epidemiologic evaluation. Baseline exams take approximately four and one-half hours with subsequent exams lasting about two and one-half hours. Due to the time commitment required for these examinations, a monetary incentive is provided upon completion of exams with a bonus at the end of each year in the study. In addition to twice yearly examinations, participants are asked about their health status during biweekly interviews. If they report illness, the symptoms are recorded.

The neurocognitive test battery includes both technician-administered, self-administered and computerized testing using the Neurobehavioral Evaluation System (NES) (Letz 1991). It is designed to look at multiple domains of neurocognitive function, including verbal and motor skills, memory, attention and spatial reasoning. The battery of tests utilized was decided upon after consultation between neuropsychologists working on the studies in North Carolina, Maryland and Virginia. Although there are slight differences in the tests used in each state, the core battery is identical so that results can be compared between the three studies.

In the event of a possible PLO-related fish kill, high numbers of fish with characteristic lesions or identification of *Pfiesteria piscicida* in Virginia waters, participants identified as being exposed to the area of concern will be requested to undergo additional examinations. Controls, matched by ten-year age group, gender and education, who were not on waters with dead fish or *Pfiesteria* will be examined at the same time.

Environmental Studies.

Personnel at DEQ, VIMS and ODU are gathering data regarding water quality and fish health (Everton et al., 1999). DEQ staff obtain water samples for quality testing and PLO examination from 14 sampling sites along the western and eastern sides of the Bay every two weeks. VIMS personnel conduct sampling of fish to determine fish health in those same areas on the same day that DEQ collects samples. Water samples are taken to ODU for screening for PLOs and SEM examination if a high concentration of PLOs is found. Personnel from DEQ and VDH's Shellfish Sanitation Program collect additional water samples monthly that are tested for quality and examined for PLOs by researchers at ODU.

Data Analysis.

For data analysis, exposure groups were created using data gathered from the biweekly interviews and 1998 environmental monitoring. Persons reporting activity on waters that were positive for PLOs in more than 50% of biweekly samples made up the PLO-exposed group; those who did not report activity in this area were non-PLO-exposed. Two variables were created to look at this exposure, one was a group variable where participants reporting an average of one hour or more per two weeks in a PLO-exposure area were considered PLO-exposed. The second was a numeric variable reflecting average time spent in PLO-exposure waters; participants who were never on PLO-exposure waters were given a value of zero.

TABLE 1. Demographics of Study Participants

	Eastern Shore # (%)	Western Shore # (%)	Total # (%)
Females	8 (19%)	11 (16%)	19 (17%)
Males	34 (81%)	59 (84%)	93 (83%)
Total	42 (100%)	70 (100%)	112 (100%)
Average Age	42.8	47.6	45.8

Distribution of Occupation Among EAS Cohort

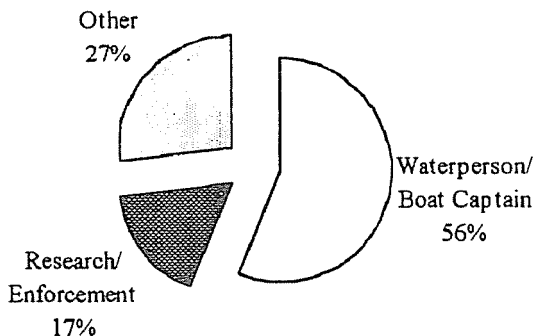


FIGURE 2. Distribution of occupations among participants in the EAS cohort study.

Parametric methods including analysis of variance, multivariate analysis of covariance and backward elimination multiple regression methods were used to look for associations between cohort characteristics or exposures and outcomes. A *p*-value of less than 0.05 was considered statistically significant.

RESULTS

Since the study began there have been 118 persons enrolled, 112 of whom are active in the project at this time. Two participants were found to be unable to conduct all the tests due to severe health problems and four others were lost to follow-up.

Eighty-three percent of current participants are males (93/112), slightly over one-third of the group lives on the Eastern Shore of Virginia (42/112) with the rest living in counties along the western side of the Bay (70/112) (Table 1). Persons living on the Eastern Shore are somewhat younger than those on the western side with an average age of 42.8 years compared to 47.6 years, respectively. Half of the cohort are professional waterpersons (51%), 17% are on the water due to research or enforcement, 5% have occupations with other water exposures and 27% have non-water-associated occupations (Figure 2). Participants in occupations not involving water spend time on the water due to recreational activities.

TABLE 2. Distribution of Water Exposure

	May – Oct 1998 # Mean Hours (Range)	Nov 1998 – Mar 1999 # Mean Hours (Range)
≥ 16 hrs/2 weeks	38 46.4 (17-116)	19 36.7 (17-80)
< 16 hrs/2 weeks	25 4.2 (0-14)	44 3.5 (0-15)

Exposure Data. During the biweekly interviews, participants report which quadrants on the EAS Study Map they were in during the previous two weeks and average time spent in each. These data are used to group the cohort based upon exposure locations and time. During the spring to fall 1998 time period, 38 of 63 participants reported being on the water more than 16 hours every two weeks. This group had an average water exposure time of 46.4 hours every two weeks (range 17-116/2 weeks) (Table 2). A comparison of reported water exposure data between the summer and winter period was done and 19 participants who spent more than 16 hours per two weeks on the water during the summer of 1998 reported fewer exposure hours during the winter months (Table 2).

When location of exposure was analyzed, it is seen that 30% of participants were active in waters along the western side of the Bay only, 39% in areas along the Eastern Shore only (either seaside or Bayside) and 28% reported activity in both areas.

Environmental Findings.

Environmental monitoring of Virginia waters occurred from June to September 1998. During that time no *Pfiesteria piscicida* was found although numerous sites were found to have other PLOs (Marshall et al., 1999). PLO counts ranged from 0-370 cells per ml. Some sites had PLOs in their samples routinely while they were rarely or never observed from other sites. Nine sampling sites along the western side of the Bay had PLO counts greater than zero more than 50% of the time, with average counts of 15 to 85 cells per ml (range 0-370). Along the Eastern Shore, PLOs were seen in only 19% to 36% of samples with average counts of 3-6 cells per ml (range 0-90). The PLO counts were slightly correlated with dissolved oxygen ($R=0.38$, $p<0.0001$), indicating that as dissolved oxygen increased so did PLO counts.

The sites where PLOs were seen in more than 50% of biweekly samples are shown in Figure 1. These sites were clustered in the northwest portion of our study area and activity on waters in the northwest quadrants surrounding the identified sites was used to determine PLO-exposure. Twenty-seven of 63 participants were exposed to this area an average of one hour or more every two weeks, with average times ranging from one hour to 116 hours per two weeks. For further data analysis, these 27 participants were considered PLO-exposed.

Medical Examinations.

Currently three rounds of medical exams have been conducted. The first round was done between April and June 1998, the second round in October and November of

TABLE 3. Visual Contrast Sensitivity by Exposure, Fall 1998

Spatial Frequency	PLO-Exposed N=27		Non-PLO-Exposed N=36		T-Score	P-Value
	Mean	SEM*	Mean	SEM*		
1.5	73.35	4.11	81.78	3.62	1.53	0.132
3	112.26	7.74	130.53	5.23	2.02	0.047
6	107.53	8.41	131.43	6.20	2.34	0.023
12	47.15	5.66	65.98	4.33	2.69	0.009
18	23.65	3.39	32.92	3.16	1.98	0.052

Source	Degree of Freedom	F-Score	P-Value
Group	1, 61	6.84	0.0112
Spatial Frequency (SF)	4, 58	235.35	0.0001**
Group*SF	4, 58	1.76	0.1498**

*SEM = standard error of the mean. **From Multivariate analysis of variance

Visual Contrast Sensitivity by Exposure, Fall 1998

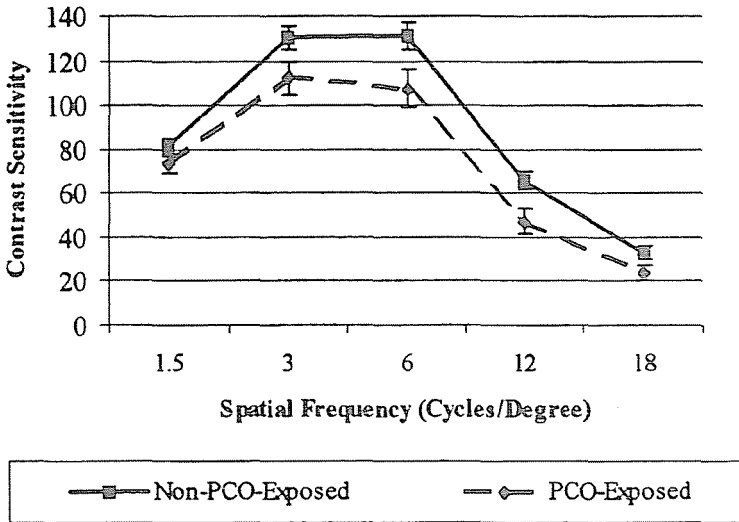


FIGURE 3. Visual Contrast Sensitivity scores from fall 1998 testing. Scores were compared between participants based on exposure to waters containing PCOs in more than 50% of samples during summer 1998.

1998 and the third round was completed in March 1999. To date, several medical conditions have been identified, including diabetes and skin cancer, but no EAS has been detected either during medical exams or biweekly interviews.

VCS testing was added to the medical examinations in the fall of 1998. In initial analysis, average VCS scores were significantly lower for the PLO-exposed group than scores for the non-PLO-exposed, with the greatest difference seen at the spatial frequency of 12 cycles per degree (VCS12) (Table 3 and Figure 3).

TABLE 4. Backward Regression Analysis of VCS12 Scores

R-square (p-value)	Variable	Parameter Estimate	Standard Error	P-value
0.4381 (0.0001)	Intercept	115.208	11.053	0.0001
	Age	-1.141	0.245	0.0001
	Average PLO- Exposure Time	-0.271	0.124	0.0340
	Cigarettes/day	-0.530	0.262	0.0478

*Predictors were eliminated if $p > 0.10$

VCS scores were compared for individuals between fall 1998 and spring 1999 (paired T-tests) to see if participants who scored below average in the fall had better scores at re-testing in the spring. No significant changes in individuals' scores were found. This indicates that once damage to this neurological-pathway has occurred, it is not reversible.

Multiple linear regression with backward elimination was performed to look at the effect on VCS12 scores of age, smoking, alcohol, average time exposed to PLO-exposure waters and average time on any water. VCS12 scores were used in the model because they showed the most significant difference between groups. Scores from fall 1998 and exposure times from May to October 1998 were used. Age was the most significant predictor of VCS12 score (Table 4) with average time in PLO-exposure waters and smoking also contributing to some of the variance in scores.

With most of the tests included in the neurocognitive battery, there is a practice effect expected on test scores, i.e., as an individual takes the same test multiple times they are expected to improve their score due to familiarity. For this study, the variable of interest in regards to the neurocognitive tests is change in score and whether or not any unusual pattern of change is seen in relationship to environmental exposures. The distribution of changes in scores from Time 1, baseline testing in spring 1998, to Time 2, fall 1998, was analyzed and no unusual trend in change was identified. Changes in scores on the neurocognitive test battery displayed a normal distribution for participants with only rare exceptions. There was no relationship identified between change in score and PLO exposure.

DISCUSSION

Although no EAS has been identified in the first year of this study, other useful data have been obtained. The environmental studies have proven very interesting and show that PLOs are normal in certain waters in Virginia. Further investigation of the common species found in Virginia waters will add to the bank of knowledge regarding the normal distribution and natural history of potentially toxic dinoflagellates.

Since no toxic PLOs were positively identified in 1998, we are still unclear as to the health affects of the PLO toxins. Although no unusual trend in change on the neurocognitive tests was seen in relationship to exposure to waters consistently containing PLOs, it is interesting that the results of contrast sensitivity testing hint at

a possible neurotoxic effect in association with chronic exposure to PLO-infested waters.

The VCS results reported here indicate that although age was the most important predictor of VCS scores, exposure to waters frequently containing PLOs contributed to the variance together with cigarette smoking. These results are somewhat different than those reported by Hudnell (1998). He examined two groups of waterpersons, those exposed to estuaries (potentially containing PLOs) and those who worked offshore exclusively. He reported VCS6 scores showed the greatest difference between these groups; and in backward elimination found membership in the estuary group to be the most important predictor of decreased VCS6 scores. Hudnell also reported that smoking, total time on the water and an age to group interaction were important predictors, so although there are differences between the two study results, there are also similarities.

It is difficult to fully interpret and understand the VCS results until we have more definitive information about the PLOs to which cohort participants were exposed in 1998. Analysis of the 1999 environmental and health data will begin once all monitoring data are compiled and fall 1999 medical data are obtained. It will be interesting to see how the 1999 water characteristics relate to participant health characteristics and if 1998 associations are repeated.

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