LONG ISLAND SOUND STUDY
EPA ASSISTANCE AWARD FINAL REPORT

1. Submission Date of Final Report to LISS: January 20, 2009

2. EPA Grant Number and Project Title: No: LI-97149601

3. Grantee Organization and Contact Name: University of Connecticut, Dr. Charles Yarish

4. Public Summary: The goal of the project was to protect guard Long Island Sound from the introduction of non-native organisms that may be imported via fishing bait worms and the seaweed packing material known as wormweed (*Ascophyllum nodosum*). The project examined bait for non-native invertebrate animals, macroalgae (also known as seaweeds), and harmful, toxin-producing microalgae. Bait was purchased from retail bait shops at locations ranging from Groton, CT in northeastern Long Island Sound to Glenwood Landing (NY) on Long Island in the southwest of the Sound. Using a combination of visual and microscopic inspection, and sophisticated molecular biological techniques to detect the presence of microalgal cells, the study questioned whether (i) non-native organisms were being imported via bait worms, and if so whether; (ii) non-native organisms vary according to purchase location, or; (iii) time of year.

Overall, 14 species of macroalgae, two species of harmful microalgae (*Alexandrium fundyense,* and *Pseudo-nitzschia multiseries*), and 23 different categories of invertebrate animals were discovered among the wormweed. Only one of the microalgal species was not native to Long Island Sound. Overall, location (eastern vs. western, northern vs. southern Long Island Sound) did not affect the number of algal or invertebrate species. Temperature did affect algal diversity and abundance, however, both in post-collection incubation (5° < 15° = 25°) and seasonally (summer produced highest numbers). Invertebrates were most abundant in summer as well.

The Gulf of Maine now harbors a diverse suite of non-native organisms. These may be exported to other areas of the U.S. via national bait wholesalers and cause ecological harm to the receiving ecosystem. In addition to potential ecological impacts associated with the import of non-native organisms, economic harm is also possible. For example, commercial shellfishing beds may be closed when harmful microalgae bloom in coastal waters. With ca. 470 retail bait shops in NY and CT, the chances of introduction of harmful non-natives is not trivial. For example, in our 18 month study of four locations, we discovered the harmful non-native microalga *Pseudo-nitzschia multiseries* in 58% of our samples.

5. Project Period: 6/1/2006- 6/31/2009

6. Project Description: **Multi-Component Evaluation to Minimize the Spread of Aquatic Invasive Seaweeds, Harmful Algal Bloom Microalgae, and Invertebrates via the Live Bait Vector in Long Island Sound**. The introductions of non-indigenous species to the Long Island Sound (LIS) have the potential to dramatically affect both the environment and economy of the area. One vector of these species yet to be examined

completely is bait worms packaged with the seaweed *Ascophyllum nodosum*. This seaweed can contain adults, juvenile, and/or reproductive bodies of invasive marine organisms. Fishermen often discard the seaweed into LIS upon using the worms. This study investigated whether invasive macro-algae, micro-algae or invertebrates are being introduced to LIS through this vector.

7. Activities & Accomplishments: Within the general goal of preventing introductions of non-indigenous species (NIS) and harmful algal bloom forming microalgae (HAB), this proposal examined bait worms as a vector for economically and ecologically harmful species. To evaluate bait worms as a vector, we successfully tested the following specific hypotheses:

Hypothesis 1a H_0: Worm baits sold along Connecticut and New York shores of LIS do not contain NIS seaweeds or HAB microalgae; if baits and seafood from LIS are discovered to contain NIS seaweeds and/or HAB microalgae:
Hypothesis 1b H_0: Worm baits sold in LIS contain similar taxonomic suites of NIS seaweeds and potential HAB microalgae
Hypothesis 1c H_0: Worm bait vectors show no seasonality in associated NIS seaweed flora or HAB microalgae

Hypothesis 2a H_0: Worm baits sold along Connecticut and New York shores of LIS do not contain non-native invertebrate animals; if baits and seafood from LIS are discovered to contain non-native invertebrate animals:
Hypothesis 2b H_0: Worm baits contain similar taxonomic suites of non-native invertebrate animals
Hypothesis 2c H_0: Worm bait vectors show no seasonality in associated non-native invertebrate animals.

The project was successfully completed, with tests of all hypotheses. The results can be used by resource managers to inform the development of policies for the bait industries to reduce or eliminate the threat of the introduction of NIS by this vector.

8. Modeling: N/A

9. Summary of Findings:
Macro- and Microalgae: Project sampling began on June 5, 2007. Boxes of bait were purchased in New York (Ebb Tide Bait and Tackle, Port Chester and Duffy's Bait and Tackle, Glenwood Landing) and Connecticut (Fisherman's World, Norwalk and Ken's Tackle, Groton, CT). On those occasions when Ebb Tide could not provide bait, samples were obtained from City Island Bait and Tackle, Bronx, NY (four times), from Pet Planet, New Rochelle, NY (once), and Sportsmen Den, Greenwich, CT (once). On January 8, 2008, Ken's Bait and Tackle burned completely. We replaced Ken's with Captain Bruce's Bait and Tackle (Groton, CT).

Sand worms (*Nereis virens*) were purchased from four retail shops from New York (NY) and Connecticut (CT) on each sampling date, with the exception of the last date in 2007 (during which only three shops were sampled). Eleven shops were sampled throughout the study (Table 1), and an attempt was made to sample from two shops in CT and two in NY on each date, although there were exceptions (Table 2). To study seasonal variations in the potential of spreading macro- and micro-algae to LIS, sampling was

conducted twice a month during the main fishing season (May-Nov) and once a month during the beginning and end of the fishing season (Table 2). Bait worms were purchased in six ½ dozen boxes or 3 one-dozen boxes, depending on availability. When possible, the baits were purchased on the same day, however sometimes extenuating circumstances sometimes required bait to be purchased the day before scheduled (Table 2, marked by an superscripted ***a***). In these cases, the bait was kept in the original boxes at 5°C until the following day. On one occasion, the retail shop did not have *N. virens*, so bloodworms (*Glycera dibranchiata*) were substituted because they are also packaged with *Ascophyllum nodosum* (Table 2, marked by a superscripted ***b***).

The baits were first taken to the Marine Biotechnology Laboratory, University of Connecticut, Stamford, CT, USA for processing and systematic evaluation. The bait worms were removed from the packaging seaweed *A. nodosum*, and the fresh weights of the seaweed and associated marshgrass (*Spartina alterniflora)* were made throughout the study to determine if there were large differences in quantity between bait shops and sampling dates. At each sample, a voucher specimen of *A, nodosum* (and *Fucus* sp.) was pressed and saved to document the morphotype found at each site. The *A. nodosum* and *S. alterniflora* were then divided between the two different aspects of the study: macroalgae and microalgae.

Table 1: Retail shops, locations, and acronyms used throughout the study.

	Retail Shop	Acronym	Latitude/Longitude
CONNECTICUT	Ken's Tackle (Groton, CT)	KT	41°20'N, 72°30'W
	Captain Bruce's (Groton, CT)	CB	41°20'N, 72°30'W
	Fishermen's World (Norwalk, CT)	FW	41°6'N, 73°24'W
	River's End (Old Saybrook, CT)	RE	41°17'N, 72°21'W
	Fish Tails (Stamford, CT)	FT	41°5'N, 73°34'W
	Sportsmen's Den (Greenwich, CT)	SD	41°30'N, 73°39'W
NEW YORK	Ebb Tide (Port Chester, NY)	ET	41°N, 73°39'W
	Duffy's Bait and Tackle (Glenwood Landing, NY)	DBT	40°49'N, 73°38'W
	Pet Planet (New Rochelle, NY)	PP	40°55'N, 73°47'W
	Jack's Bait and Tackle (Bronx, NY)	JT	40°51'N, 73°52'W
	City Island (Bronx, NY)	CI	40°51'N, 73°52'W

Table 2. Sampling dates and sites.

		Eastern LIS (CT)			Western LIS (CT)			Far Western LIS (NY)				
#	Sampling Date	KT	CB	RE	FW	FT	SD	ET	CI	DBT	PP	JT
1	June 5, 2007	X			X			X		X		
2	June 18, 2007	X			X		X			X		
3	July 2, 2007	X			X			X[a]		X		
4	July 19, 2007	X[a]			X			X		X		
5	August 8, 2007	X			X			X[a]		X		
6	August 23, 2007	X[a]			X			X		X		
7	September 10, 2007	X[a]			X					X	X	
8	September 24, 2007	X			X				X	X[b]		
9	October 8, 2007	X			X				X	X		

10	October 22, 2007	X			X				X	X		
11	November 5, 2007				X			X[a]		X		
12	April 22, 2008		X		X	X[a]				X[a]		
13	May 12, 2008		X		X	X[a]				X[a]		
14	June 2, 2008			X	X	X[a]				X		
15	June 16, 2008		X		X				X	X		
16	July 7, 2008		X		X				X	X		
17	July 22, 2008		X		X					X		X
18	August 4, 2008		X		X					X		X
19	August 18, 2008		X		X				X	X		

[a]samples purchased the day before; [b]3 dozen bloodworms were purchased

A summary of common epiphytes and endophytes found associated with *Ascophyllum nodosum* along the coastline of Maine was also produced from the University of New Hampshire Albion R. Hodgdon Herbarium (see Appendices 1-3). The *A. nodosum* collected in the present study was first examined to determine whether any of these epiphytic or endophytic macroalgae could be found prior to incubation. Portions of the seaweed were removed and cultured to promote the growth of these macroalgae: at least three pieces of thalli from each of the basal, apical, and branch portions of the *A. nodosum* were included in 200ml of enriched von Stosch (VSE) media in a deep Petri dish (Ott 1965). In addition, any *S. alterniflora* or seaweed species (mainly, *Fucus* sp.) that were mixed with the *A. nodosum* in the bait boxes were also included. Dishes were cultured for 10 d in one of three temperatures (5, 15, 25°C) under a 12:12 L:D photocycle with a photon flux rate of 40 µmol photon $m^{-2}s^{-1}$. The thalli sections were then examined for the growth of epiphytic or endophytic associated marine macroalgae. If a positive identification could not be made at that time, the material was placed back into culture until the morphological identification was possible. Two identification keys were utilized throughout the analysis (Villalard-Bohnsack 1995, Sears 2002). During the later part of the study, notes were also kept on whether or not these epiphytes were found on the *A. nodosum* or on *S. alterniflora.*

For the microalgae, approximately 1/3 of the seaweed packaging and associated cordgrass from each sampling site was added to an Erlenmeyer flask containing 500ml of 0.45µm-filtered, autoclaved seawater and shaken to release any adherent microalgal cells, i.e. vegetative cells or cysts. This seawater then was sieved through a 50-µm filter to, and the filtrate was distributed into 50-ml conical tubes. One tube was preserved with Lugol's solution for subsequent microscopic identification, three tubes were used for culture purposes, and two-four tubes were used for DNA extraction. These DNA samples are termed the "initial" samples. The day following the sampling date, the 50ml tubes which were labeled for culture were added to 200ml F/2 media (Anderson 2005) in a 250-ml tissue culture flask. The flasks then were incubated for 10 d at the same temperatures as the macroalgae (5, 15, 25°C) but under a light intensity of 80 µmol photon $m^{-2}s^{-1}$. After incubation, the contents were prepared for DNA extraction. The DNA samples that were extracted after the incubation period are termed "post" samples.

One to two-hundred milliliters of each initial rinse sample and 50 ml of each post-culture incubation medium sample were centrifuged at 4000g for 10 min. The supernatant was removed, and the pellet was re-suspended in approximately 1 ml of residual liquid. This concentrated sample was transferred into a 1.5-ml tube, and centrifuged at 12,000 rpm for 3 min. The pellet was suspended in DNA lysis buffer (10 mM Tris pH 8.0, 100 mM EDTA pH 8.0, 0.5% SDS, 200 µg/ml proteinase K), and DNA extractions performed using a CTAB protocol (Zhang and Lin 2005) for the 2007 samples. In one case (100807), the

supernatant was extracted using phenol-chloroform before proceeding to the Zymo DNA Clean and Concentrator kit (Zymo Research; Orange, CA). At the end, the DNA was eluted with 80 µl 10mM Tris•HCl. DNA concentration and quality were measured spectrophotometrically using a Nanodrop (Thermo Scientific; Waltham, MA). DNA quality was further examined by PCR using a universal 18S rDNA primer set (see below). If the PCR failed, the DNA solution was extracted again with phenol-chloroform and run through the Zymo column again.

Despite the extensive efforts to obtain PCR-amplifiable DNA, some of the samples failed in PCR, particularly with the "initial" samples, most likely due to inhibitory compounds from the mud and debris associated with the *A. nodosum,* which were rich in phenolic compounds. To alleviate this problem, a Soil Microbe Kit (Zymo Research; Orange, CA) was utilized to extract DNA in 2008. The samples were centrifuged as above; however, with this method, the pellet was added to the kit's lysis buffer and homogenized at 6.5 m/s for 45 seconds. The kit protocol was followed, continuing through the last step of centrifugation through the IV-HRC spin filter. DNA was eluted with 100 µl of the elution buffer provided.

PCR was run on the samples to determine if particular target species were present. First, a set of universal 18S rDNA primers was used to determine which samples contained amplifiable DNA. PCR inhibitors were often found within the samples; therefore, this amplification was critical in ensuring no false negatives (Lin 2008). Once the DNA was deemed clean enough to amplify, PCR was run for seven individual species. Specifically, six dinoflagellates (*Alexandrium fundyense* Balech, *Karlodinium veneficum* (D. Ballantine) J. Larsen, *Pfiesteria piscicida* K.A. Steidinger & J.M. Burkholder , *Pseudopfiesteria shumwayae* (Glasgow & Burkholder) Litaker, Steidinger, Mason, Shields & Tester , *Akashiwo sangiunea* (K. Hirasaka) G. Hansen & Ø. Moestrup , *Karenia brevis* (C.C. Davis) G. Hansen & Ø. Moestrup) and one diatom (*Pseudonitzschia multiseries* (Hasle) Hasle) were targeted for occurrence.

Both the universal 18S rDNA and *Alexandrium*-specific PCRs were run using Takara Hot Start Ex Taq system with 1 µl DNA. 18S rDNA amplification was done in 35 cycles of 95°C for 25 sec, 56°C for 30 sec, 72°C for 40 sec, followed by an additional extension step at 72°C for 5 min. For *A. fundyense*, the cycle program was 35 cycles of 95°C for 20 sec, 58°C for 25 sec, 72°C for 30 sec, followed by a final step of 72°C for 5 min. PCR for other target species was run through a Bio-Rad iQ iCycler system (BioRad; Hercules, CA) to achieve higher through-put. Reaction was assembled using 8 µl of iQ SYBR Green Supermix (BioRad; Hercules, CA), with 8 µl of 8-fold diluted DNA (equivalent to 1 µl of original DNA), and 0.5 µl of each of the two primers. The Real-Time PCR program included an initial denaturation at 95°C for 3 min, 40 cycles of 95°C for 15 sec, 58°C for 25 sec, and 72°C for 20 sec, and then a melting curve analysis run from 55°C to 95 °C. The annealing temperatures can be found in Table 3 for each individual PCR reaction. To validate the positive results for targeted species, the PCR products were cloned and sequenced for comparison with known sequences reported in GenBank.

The samples preserved with Lugol's were kept in the dark at 4°C until analysis. A 1-ml sample was placed on a Sedgewick Rafter slide and observed using an Olympus BX51 compound microscope. Photomicrographs were taken of the representative species with a Q-imaging QiCam camera through a long-working- distance, 40x objective. Algae were identified to genus or species for on the most prevalent species present, and the rest were grouped according to taxonomic class.

After analyzing the Lugol's preserved samples, two were chosen to have DNA cloned and sequenced. The purpose was to look more broadly for potential HAB and other

microalgal species that might escape microscopic analysis because of low abundance or small cell size, or molecular detection because they were not one of the target species. One sample for this analysis was taken from the fall of 2007 and the other was from the early summer of 2008. These samples were chosen because they contained a wide range of organisms as found microscopically. The DNA was purified and amplified for 18S rDNA as described above. The product was run on a 1% agarose gel, and the band was excised under UV light. DNA was extracted from the gel using a Gel DNA Recovery Kit (Zymo Research). The purified product underwent a Taq treatment to ensure the presence of an A overhang (10 µl DNA, 1 µl dNTP, 1 µl 10x buffer, and 0.1 µl Ex Taq, incubated at 72°C for 15 minutes) and was ligated into a T-vector (Takara) according to the manufacturer's instructions. After transformation into XL-1 competent blue cells (Invitrogen), 120 colonies were randomly picked and grown overnight for plasmid isolation. The plasmids verified to contain inserts were sequenced using T7 and T3 primers from a Big Dye Terminator v3.1 cycling kit (Applied Biosystems) on an ABI Prisom automated sequencer at the Yale DNA Facility.

To determine whether the data indicated a significant difference between sampling sites and incubation temperatures, statistical methods were used. A t-test was run to establish if there were differences between samples collected from New York vs. Connecticut, between retail sites on the northern shore of LIS vs. the southern shore, and between the eastern end of LIS vs. the western end. A one-way ANOVA was also used to test whether the 10-day incubation produced a larger number of species found as compared to the initial sampling date data. Finally, incubation temperature and season were tested for effects upon the total species number by a two-way ANOVA to determine if seasonality could be a risk factor for an introduction to occur.

The mass of the *A. nodosum* ecad *scorpioides* and *Spartina alterniflora* in the pooled bait containers was weighed throughout the study (Fig. 1). Overall, there were no significant differences between sampling dates or between sampling sites for each date ($p>0.05$, one-way ANOVA). On each sample day, *A. nodosum* was examined for epiphytic or endophytic algae. On only one date was anything found pre-incubation on the seaweed: *Cladophora ruchingeri* Kützing was epiphytic on the *A. nodosum* on July 2, 2007 from site DBT. Two species of *Fucus* were found occasionally mixed within the *A. nodosum* on the day of sampling: *F. vesiculosus* Linnaeus and *F. spiralis* Linnaeus (Table 4). These seaweed species were also found to have epiphytic macro-algae after the incubation was complete.

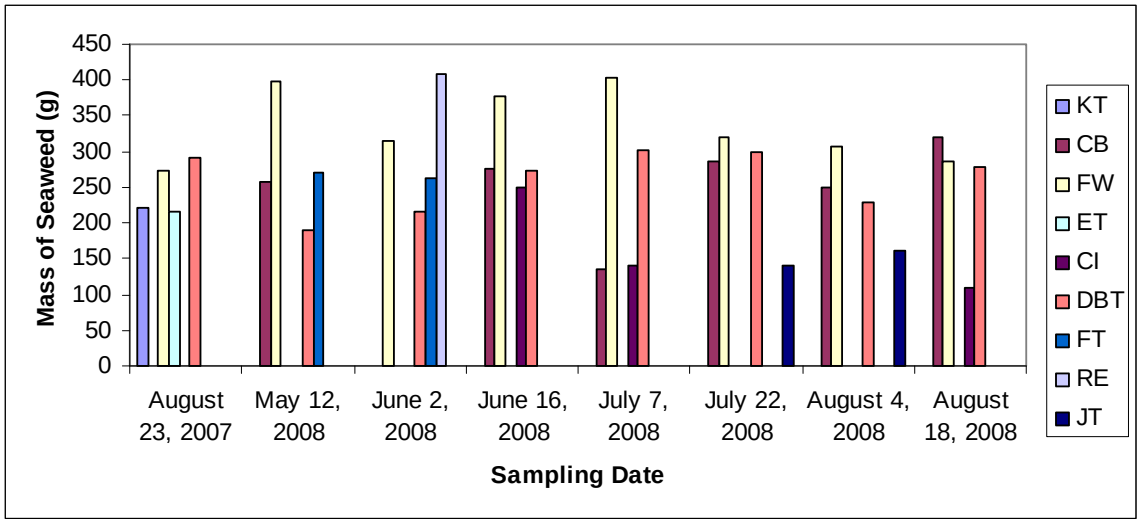

Fig. 1: Seaweed mass variation measured throughout the sampling period (see Table 1 for sample-location codes).

After incubation, the samples were re-inspected for the presence of other macro-algae. Throughout this study, a total of 13 different macroalgal species were found within the cultures (Table 5): *Chaetomorpha linum* Kützing, *Cladophora ruchingeri* Kützing, *Ectocarpus siliculosus* Lyngbye, *Myrionema corunnae* Sauvageau, *Percursaria percursa* Bory de Saint-Vincent, *Pilayella littoralis* (Linnaeus) Kjellman, *Rhizoclonium tortuosum* (Dillwyn) Kützing, *Ulothrix flacca* (Dillwyn) Thuret, *Ulva clathrata* Le Jolis, *Ulva compressa* Agardh, *Ulva flexuosa* (Agardh) Wynne, *Ulva intestinalis* (Linnaeus) Link, and *Ulva prolifera* O.F. Müller. All of these are currently present in LIS (Schneider *et al.* 1980, Keser *et al.* 2006).

Five different *Ulva* species (including tubular and blade) were also found, though these required incubation longer than 10 d to identify. Often, *Ulva* was found, but it did not survive in culture to the point of species identification (indicated on the last line of Table 5). Throughout the study, notes were kept on whether the epiphyte was found on *Ascophyllum nodosum* or on the associated *Spartina* sp. Overall, epiphytes and endophytes were found twice as frequently on *Spartina alterniflora* as on *Ascophyllum nodosum*..

Table 4: Dates, sites, and species of *Fucus* included in the bait-worm packaging (see Table 1 for sample-location codes). Fv: *Fucus vesiculosus*; Fs: *Fucus spiralis*

	Sample Site					
	CB	**FW**	**SD**	**FT**	**DBT**	**ET**
5-Jun-07					**Fv**	
18-Jun-07			Fs **Fv**			
2-Jul-07					**Fv**	
19-Jul-07		Fs				
8-Aug-07		Fs				Fs
22-Oct-07		Fs			Fs	
5-Nov-07		Fs				Fs
22-Apr-08	Fs	Fs		Fs	Fs	
12-May-08	Fs					
16-Jun-08					Fs	
7-Jul-08	Fs	Fs			Fs	

Overall, 70% of the project's microalgal DNA samples were clean enough to proceed with the species-specific PCR's. Seven HAB species were targeted molecularly with PCR: *Alexandrium fundyense*, *Karlodinium veneficum*, *Pfiesteria piscicida*, *Pseudopfiesteria shumwayae*, *Akashiwo sanguinea*, *Karenia brevis,* and *Pseudo-nitzschia multiseries*. Two of these species were found throughout the study: *Alexandrium fundyense,* and *Pseudo-nitzschia multiseries* (Table 6). The lower limit of the PCR system can detect DNA sequences from as little as 0.1 cell/ml of sample. Sequences obtained from randomly-selected PCR confirmed that they were, indeed, the target species.

The rinse samples preserved with Lugol's solution revealed a diverse microalgal community. Genera found consistently throughout the study included diatoms such as *Cocconeis* Ehrenberg*, Thalassiosira* Cleve*, Chaetoceros* Ehrenberg*, Navicula* Bory de Saint-

Vincent, and *Cylindrotheca* Rabenhorst, *Caloneis* Cleve, *Melosira* Agardh, and *Nitzschia* Hassall (Table 6). Of the samples examined, DBT 5°C from October 22, 2007 and FW 15°C from June 2, 2008 contained a wide array of organisms and thus were selected for further molecular analysis. Based on the 90 clones sequenced, the DBT sample contained a large community of diatoms, with *Skeletonema* spp. accounting for approximately 70% of the microalgae within the samples (Fig. 2A). The next dominant genera included *Thalassiosira* and *Nitzschia*. Of the 102 clones from the FW sample, however, the sequences showed a mixture of both ciliates and diatoms (Fig. 2B), with *Euplotes* being the most dominant lineage, followed by *Navicula, Nitzschia, Holosticha,* and *Diophrys*. Together, the sequencing data, combined with the microscopic examinations of the Lugol's-preserved samples, indicate there is a highly diverse protist community contained within the bait-worm packaging.

Table 5: Summary of macroalgae found post-incubation.

	2007											2008							
Species	5-Jun	18-Jun	2-Jul	19-Jul	8-Aug	23-Aug	10-Sep	24-Sep	8-Oct	22-Oct	5-Nov	22-Apr	12-May	2-Jun	16-Jun	7-Jul	22-Jul	4-Aug	18-Aug
Chaetomorpha linum						●													
Cladophora ruchingeri			●							●	●		●				●	●	
Ectocarpus siliculosus				●	●		●				●	●		●	●		●		●
Myrionema corunnae	●														●		●		
Percursaria percursa	●			●			●		●		●			●					●
Pilayella littoralis									●										
Rhizoclonium tortuosum	●	●	●	●		●	●	●	●			●	●	●	●		●	●	●
Ulothrix flacca	●	●	●	●	●	●			●		●	●	●		●				
Ulva clathrata						●	●		●		●		●						
Ulva compressa								●	●			●			●				
Ulva flexuosa						●	●		●			●		●					
Ulva intestinalis	●			●	●	●	●		●	●	●	●	●	●	●			●	●
Ulva prolifera														●					
Ulva distromatic blade	●		●	●	●	●	●	●			●		●	●	●	●	●		●

Table 6. Summary of targeted microalgal samples found pre- and post-incubation.

	2007											2008							
Species	5-Jun	18-Jun	2-Jul	19-Jul	8-Aug	23-Aug	10-Sep	24-Sep	8-Oct	22-Oct	5-Nov	22-Apr	12-May	2-Jun	16-Jun	7-Jul	22-Jul	4-Aug	18-Aug
A. sanguinea																			
A. fundyense	●		●	●			●					●		●	●	●	●	●	●
K. brevis																			
K. veneficum																			
P. piscicida																			
P. shumwayae																			
P. multiseries	●	●	●	●	●	●	●	●		●		●		●	●	●	●	●	●

Table 6: Microalgal species found within the Lugol's-preserved samples.

	2007										2008							
Species	18-Jun	2-Jul	19-Jul	8-Aug	23-Aug	10-Sep	24-Sep	8-Oct	22-Oct	5-Nov	22-Apr	12-May	2-Jun	16-Jun	7-Jul	22-Jul	4-Aug	18-Aug
Caloneis sp.	●	●	●	●	●	●	●	●			●		●	●	●		●	
Chaetoceros sp.		●					●		●			●			●			
Cocconeis sp.	●	●	●	●	●		●	●	●	●	●	●	●		●	●	●	●
Cylindrotheca sp.		●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
Melosira sp.	●	●	●					●		●		●		●		●		
Navicula sp.	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
Nitzschia sp.	●	●	●	●	●	●	●	●	●	●		●	●	●	●			
Thalassiosira sp.	●	●	●	●	●	●	●		●		●	●	●	●		●	●	●

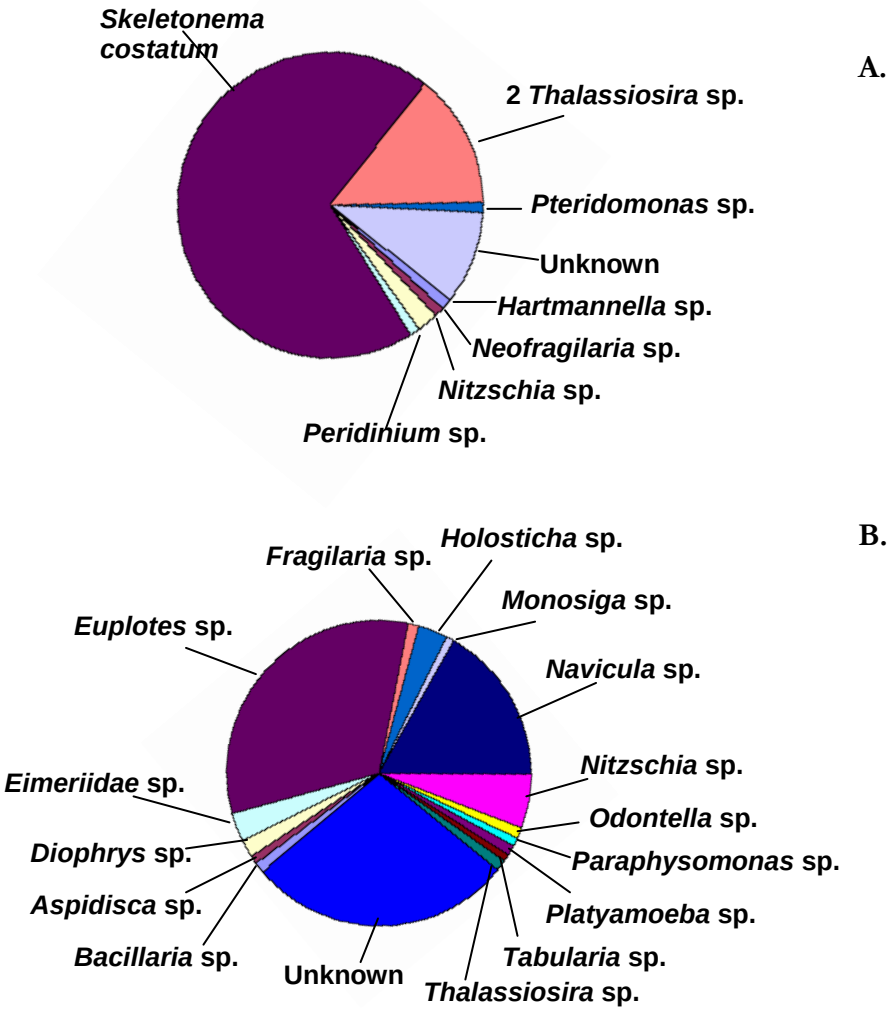

Figure. 2: Microalgal species found through DNA sequencing in DBT 5 (A) and FW15 (B).

There were no significant differences ($p > 0.05$) in total number of algal species (seaweed and microalgae combined) between sites in New York vs. those in Connecticut, between northern sites (all excluding DBT) vs. southern sites (DBT), or between eastern sites (KT, CB, and RE) vs. western locations (all remaining sites). The 10-day incubation increased the number of epiphytes found; significantly more species were found after the incubation period for both years ($p < 0.001$). One-way ANOVA for both the HAB and macroalgae species showed no effect of incubation temperature on the number of HAB species found ($p > 0.05$). However, temperature had a strong effect on the number of macroalgal species detected ($p < 0.001$); the 5°C incubation produced fewer species (avg = 0.5 species) than the 15°C (avg = 1.3 species) and 25°C (avg = 1.5 species) incubations. A two-way ANOVA revealed no interaction between sampling date and incubation temperature on the number of total algal species found (macro- and micro-algae combined). . A one-way ANOVA test revealed that no incubation temperature was more likely to produce HAB-forming species ($p > 0.05$).

Invertebrate Animals: For each sampling date, contents of all six boxes obtained from each tackle shop were combined into one container and evenly divided up between macro-algae analysis, the micro-algae analysis, and the invertebrate analysis. The seaweed was examined for invertebrates and any dislodged invertebrates were collected from the tray and preserved in a 70% ethanol solution until identification. Several weeks following preservation, all invertebrates were identified to lowest practical taxonomic category and enumerated using a 40x dissecting microscope and relevant taxonomic keys (Gosner, 1979, Weiss, 1995) Species diversity was represented using the Shannon-Weiner index.

Nine categories of invertebrates were identified in the boxes of baitworms: isopods, amphipods, bivalves, annelids, gastropods, arachnids (mites), ostracods, copepods and insect larvae (Table 1). In general, overall species composition of invertebrates obtained from the baitworm boxes did not vary between the tackle shops. A total of 23 separate invertebrate taxa were found in the samples and all samples were typically dominated by three species: the gastropod *Littorina saxatilis*, the amphipod *Hyale nilssoni* and isopod *Jaera marina*.

The total number of individuals found in the baitworm boxes did display considerable variability between sampling locations, although there was no consistent pattern among sampling periods. In most instances, however, the highest numbers of invertebrates were recorded between the months of June and August when invertebrate abundance is generally known to be highest in coastal New England waters (Fig. 1). Species diversity estimates typically varied from 1.0 to 2.5 and there were no consistent patterns between sampling locations and sampling date (Fig. 2). Decreases in species diversity usually correlated with samples containing large numbers of *Jaera marina* during July and August (Fig. 2).

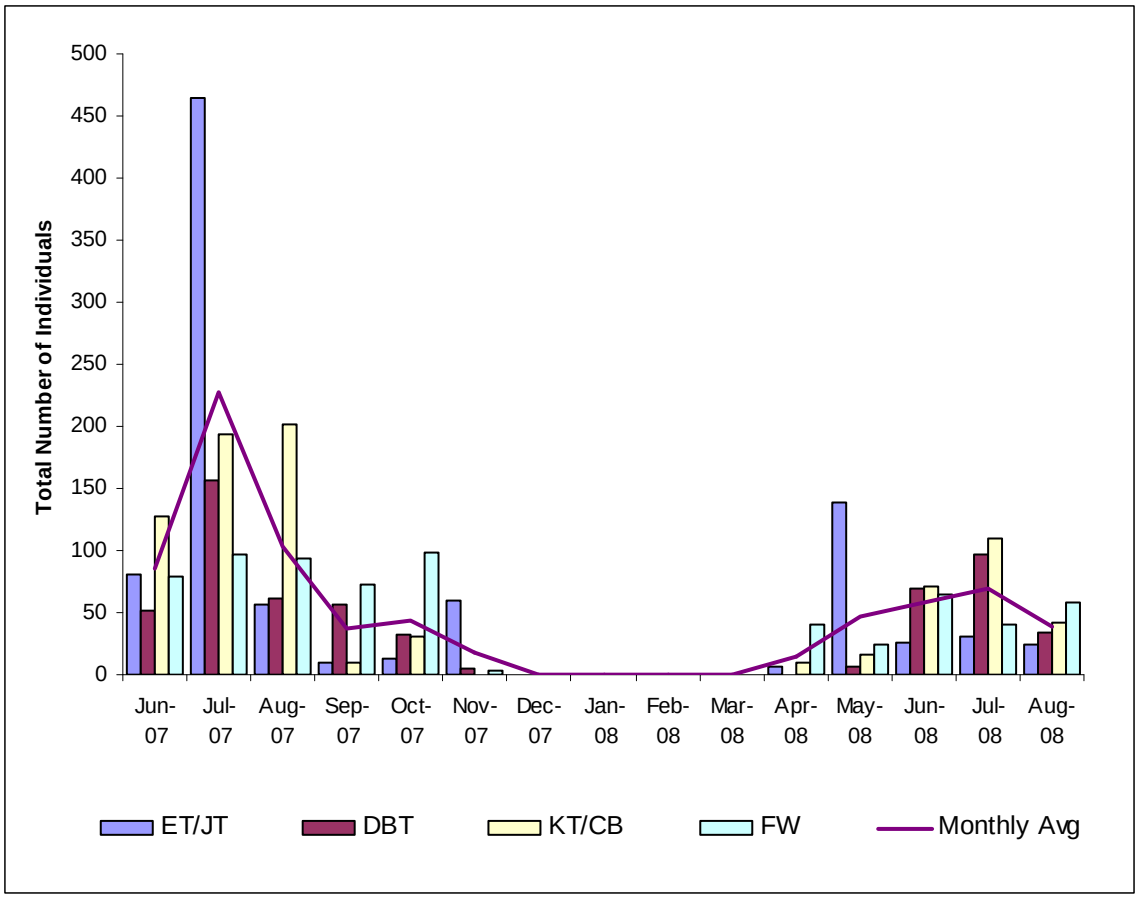

Figure 1: The total number of individuals present every month in all samples from each tackle shop. No samples were obtained between December 2007 and March 2008. The summer months appear to have the greatest number of individuals per sample than the fall months, despite the high variability between tackle shops.

Table 1: Species present in the samples collected from the four tackle shops.

		Sample Site			
	Species	**ET**	**DBT**	**FW**	**KT**
Amphipods	*Caprella penatis*	✓	✓		✓
	Eulimnogammarus obtusatas	✓	✓	✓	✓
	Gammarid Amphipod (unk)		✓	✓	
	Hyale nilssoni	✓	✓	✓	✓
	Jassa falcata				✓
Gastropods	*Hydrobia* spp	✓	✓		✓
	Littorina littorea			✓	
	Littorina obtusata	✓	✓	✓	✓
	Littorina saxatilis	✓	✓	✓	✓
Bivalves	*Gemma gemma*				✓
	Mercenaria mercenaria		✓	✓	
	Mya arenaria	✓	✓	✓	
	Mytilus edulis	✓	✓	✓	✓
Annelids	*Enchytraeus albidus*	✓	✓	✓	✓
	Oligochaete	✓	✓	✓	✓
	Spirorbis spirillum	✓			
Arachnids	Trombidiid mite	✓	✓	✓	✓
	Halacarus sp	✓	✓	✓	✓
Isopod	*Jaera marina*	✓	✓	✓	✓
Copepod	*Trigiopus*	✓	✓	✓	✓
Ostracod	*Unknown ostracod*	✓	✓		✓
Insects	Chironomid larvae	✓	✓	✓	✓
	Dipertan larvae				✓

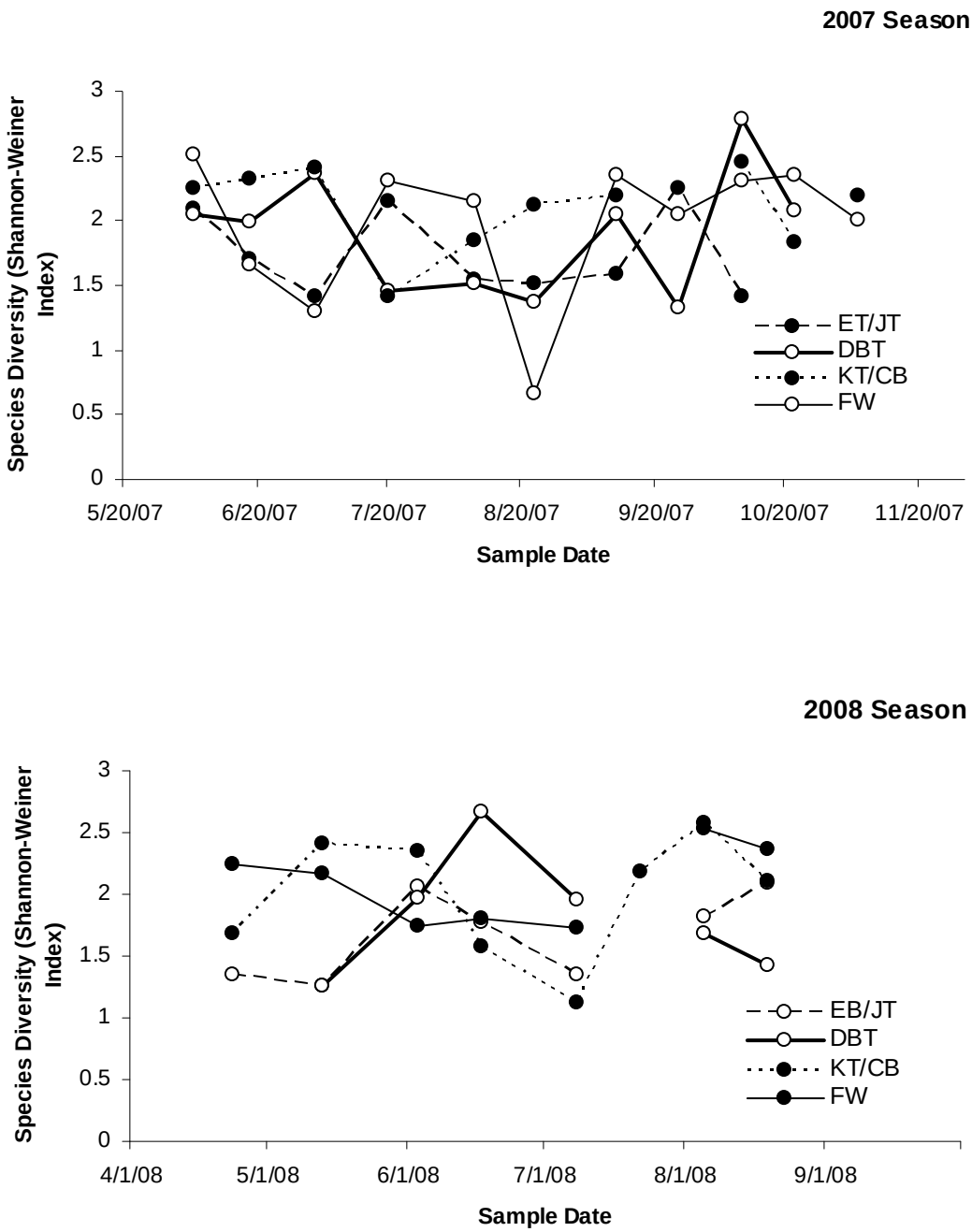

Figure 2: Species diversity calculated from the samples taken from the four tackle shops. Breaks in the data are the result of too few species in the sample to run the analysis or the inability to obtain a sample on that date. The decreases in the species diversity correlate with large numbers of *Jaera marina, Hyale nilssoni* and/and *Littorina saxatilits*. Species diversity increased in most of the 2007 samples during the fall where the abundance of the dominant species decreased substantially or they were absent from the samples.

10. Conclusions: Previous studies have shown that bait worm packaging can be a vector of non-native and potentially invasive species (Cohen *et al.* 2001, Carlton *et al.* 2001). Our study further demonstrated the extent of this threat to the Long Island Sound. Although the macroalgal species found post-incubation are all indigenous to LIS, four genera, *Chaetomorpha, Cladophora, Ulva*, and *Pilayella*, cause blooms in temperate waters (Valiela *et al.* 1997, Mathieson and Dawes, 2002). These species can shade habitat-forming benthic algae and submerged aquatic vegetation, decreasing their photosynthesis and growth (Wallentinus and Nyberg 2007). Even though the macroalgal species found within the bait worm packaging are native to LIS, this does not imply an absence of risk. Populations of the same species may be genetically distinct from each other. Thus, this vector could still be introducing non-native macroalgal ecotypes which could have a more opportunistic life history than the native ecotype, thereby having an effect on the ecosystem (Lüning, 1990, Mathieson et al. 2003).

Two harmful microalgae were found within the bait-worm packaging: *Alexandrium fundyense* and *Pseudo-nitzschia multiseries*. *A. fundyense* has formed toxic blooms in the Gulf of Maine every year (Anderson et al. 2005 and references therein). In recent years, its bloom extended southward, with severe toxic outbreaks occurring along the south shore of Long Island Sound in 2008 (http://www.longislandsoundstudy.net/newsmail/june_08_onlinev.htm). This species was detected in the bait-worm packaging throughout the study period, with higher occurrence in July and August 2008 corresponding with a PSP outbreak on August 1, 2008 that eventually closed Maine's shellfish beds (Fitzpatrick 2008). Although it is unclear how *A. fundyense* was introduced into LIS, our results indicate that bait worm products are a potential vector. It is interesting to note that the most severe bloom in LIS has occurred in Northport Harbor where fishing has been very active.

The diatom *Pseudo-nitzschia multiseries* has not yet been recorded in large numbers in LIS, but the presence of this HAB is been detected occasionally (S. Lin, unpublished observation). In our study, *P. multiseries* was detected via microscopic examination in one sample and through molecular analysis for many samples. Clearly, bait worm products could be a vector for its introduction into LIS.

There were no significant differences in the number of algal species carried by *A. nososum* bait packing between sampling sites throughout the study, indicating a similar risk of purchasing NIS-contaminated bait worms at the four sites around LIS. In addition, incubation of the samples for the 10 d period at 5, 15, and 25°C demonstrated that organisms contained with *Ascophyllum nodosum* were capable of growing under a variety of conditions. This incubation period tested the viability of the hitchhiking micro- and macroalgal species. We conclude that many are likely able grow and perhaps establish viable populations. The first stage of a successful species invasion is for the organism to arrive, survive, and establish itself within a body of water, while the second stage is to spread and affect the native species (Allendorf and Lundquist 2003). The observation that *P. multiseries* was present in the packaging and survived the 10-day incubation at LIS's range of temperatures indicate that once introduced, it can survive in LIS and the surrounding waters. If it ever forms blooms, it would exert great ecological and economic impacts on LIS because it is a toxigenic organism.

The temperature incubations demonstrated growth of macroalgal epiphytes and endophytes associated with the *Ascophyllum nodosum* over a range of temperatures that reflect the seasonal range in LIS (Pedersen et al. 2007), though the likelihood of culturing macroalgal species at 5°C was less than at the other temperatures. This suggests that the greatest threat exists during the warmer months. In fact, many of the species found in this study are eurythermal north Atlantic taxa with warm temperature affinities (Lüning 1990). This is important because the main fishing season is during the summer and fall, during which water

temperatures would be favorable for these organisms. For the HAB species, survival and growth occurred under all incubation temperatures. This could indicate that these microalgae could be introduced throughout the year. In fact, there was no significant interaction between season of sampling and incubation temperature with respect to species richness, implying that there is similar risk of introducing these species throughout the fishing season.

Our findings may have implications for other areas because Maine exports bait worms and *Ascophyllum nodosum* throughout the U.S.A. However, we recognize that the transportation process may affect the viability of the NIS and HAB organisms and should be examined. The current geographic distributions of both *Alexandrium fundyense* and *Pseudo-nitzschia multiseries* should also be taken into account when analyzing these data. *Ascophyllum fundyense* is only found in the northern hemisphere within a North American clade (John *et al.* 2003). This species is found along the northeast coast of North America. However, as the populations move towards the south, blooms become more infrequent and less toxic (Colin and Dam 2002). As a consequence, it is considered to be a cold-water species and is not expected to thrive at warmer water temperatures. In our study, *Alexandrium fundyense* was found within the 25°C incubation samples, indicating it is capable of growing at higher temperatures. Future work would need to assess the toxicity potential of *A. fundyense* at this warmer temperature. *P. multiseries,* on the other hand, is a cosmopolitan species found throughout the world's oceans at a large range of temperatures (Hasle 2002). It is present in both hemispheres and extends from the northern to the southern latitudes. As such, it is capable of surviving in a large range of environments. This is reflected in this study by *P. multiseries* presence at each incubation temperature throughout the study period.

Examination of the Lugol's-preserved samples revealed a high diversity of organisms within the *Ascophyllum nodosum* packaging material. A complex microalgal community is, therefore, transported by this vector. Many of these microalgal and protozoan species would not have been found without sequencing the samples because of their low abundance and inconspicuous habit. Although differences between these DNA samples could not be directly compared because they were from different incubation temperatures (5° and 15°C) and each underwent a different DNA-extraction method, the diverse flora found in both samples indicate the potential of introducing a complex microbial assemblage that could survive in a new environment. At present, we cannot ascertain whether any of those species may become harmful in other systems.

While bait worm boxes containing *Ascophyllum nodosum* transfer of a variety of benthic invertebrates between regions in the United States (Miller, 1969, Crawford 2001), to date, we found no NIS transferred between the Gulf of Maine and LIS. However, *J. marina*, a dominant isopod in all samples, is considered a cryptogenic species (Maclellan 2005). This species distributional range has been described as the north side of Cape Cod to Newfoundland with some southward extension, but the specific southern-most location has yet to be confirmed (Pollock 1998).

Implications. There are approximately 470 bait shops currently in Connecticut and New York States. The likelihood of a non-native species to be introduced into a habitat increases with the number of release events (Allendorf and Lundquist 2003), and recreational fishing is a very common activity within LIS. Educating both retailers and fishermen about the dangers of bait worm packaging and steps they can take to reduce the risk of invasive species introductions could have an immediate benefit to LIS (Padilla and Williams 2004). Weigle et al. (2004) surveyed bait businesses and found that 60% of retailers who import non-local bait worms receive them packaged with seaweed. They also noticed non-target (non-worm) species

included within the packaging. Yet, nearly half of those surveyed did not know of the concept of invasive species and the environmental damage they can cause. Recently, the Connecticut Sea Grant Program has begun a sticker and education programs in which bait shops receive warning stickers to place on their boxes of bait and display posters in their stores (N. Balcom, pers. comm.).

Although this study specifically targeted species which could potentially be harmful to LIS, it is important to note that bait worms are shipped from Maine to coastal locations throughout the United States and Europe. Given this, the species which were found within and on the *Ascophylllum nodosum* could potentially be dispersed to other habitats. Survival of the HAB species at different temperature in this study underlines the potential threat to different areas. Both seaweed invasions and HAB-forming phytoplankton can dramatically affect ecosystem structure and function, thus posing major challenges for coastal management of these marine habitats (Valentine *et al.* 2007). Prevention programs similar to what Connecticut Sea Grant is conducting would help reduce the probability that potential invaders could be introduced and established within coastal waters. In addition to the algae included with the samples, it is possible that the worms themselves are vectors of non-native organisms. If these were found to be carriers of harmful species, then individual states would need to assess the risk of importing these worms into their marine coastal systems. Recommendations or suggestions could be made to develop a system of certification and best practice guidelines to include guarantees that wholesalers and retailers market "invasives-free" bait-worm products and take active steps to reduce the risk of invasive species introductions (USGS 2003). In addition, other potential vectors need to be considered in taking preventative measures against invasive species. Among others, shellfish aquaculture has been found to be one of the major vectors to spread invasive organisms (Carlton 1999). This vector is very similar to bait-worm packaging in that both transport specific marine organisms across continents that may have non-native and invasive species included in the shipment.
It is noteworthy that invasive species have been introduced into Maine's coastal waters, and these have the potential to be further spread to other areas through *Ascophyllum nodosum* packaging. Among documented invaders are: the green crab (*Carcinus maenus* Linnaeus), the Asian shore crab *(Hemigrapsus sanguineus* De Haan), several tunicates (*Didemnum* Savigny sp., *Botrylloides violaceus* Oka, *Styela clava* Herdman), a green algae (*Codium fragile* (van Goor) P.C. Silva), an oyster parasite (*Haplosporidium nelsoni*), a salmon virus (*Orthomyxovirus*), and a bryozoan (*Membranipora membranacea* Linnaeus) (Thayer and Stahlnecker 2006), and even some Asiatic *Porphyra* Agardh species (Neefus *et al.* 2008). Although these species were not found throughout this study, there is the potential for these organisms to be moved throughout the country in bait-worm packaging, and additional research will be needed to address this threat. Baitworms boxes containing *Ascophyllum* is well recognized to be an important potential vector for the transfer of a variety of benthic invertebrates between regions in the United States (Miller, 1969, Crawford 2001). To date, however, no non-native species have been found being transferred in the bait boxes between the Gulf of Maine and Long Island Sound.

Summary. The results of the tests of the hypotheses are here:

1. Hypothesis 1a H_0: Worm baits sold along Connecticut and New York shores of LIS do not contain NIS seaweeds or HAB microalgae – **REJECTED; although no NIS macroalgal species were discovered in the initial inspection of the worm bait packages or after 10 d growth at 5°, 15°, or 25°C, molecular methods (and one microscope ID) detected two HAB microalgae species (*Alexandrium fundyense* and *Pseudo-nitzschia multiseries*)**

2. Hypothesis 1b H_0: Worm baits sold in LIS contain similar taxonomic suites of NIS seaweeds and potential HAB microalgae - **PROVISIONALLY NOT REJECTED; no pattern in the detection of macroalgal or microalgal species was observed as a function of bait purchase location**
3. Hypothesis 1c H_0: Worm bait vectors show no seasonality in associated NIS seaweed flora or HAB microalgae – **REJECTED; macroalgae and HAB microalgae were more frequently detected during warmer months (May-Oct)**
4. Hypothesis 2a H_0: Worm baits sold along Connecticut and New York shores of LIS do not contain non-native invertebrate animals – **NOT REJECTED; all invertebrates recorded during the study are current residents of LIS**
5. Hypothesis 2b H_0: Worm baits contain similar taxonomic suites of non-native invertebrate animals *(not relevant since no NIS invertebrates were recorded in bait purchases)*
6. Hypothesis 2c H_0: Worm bait vectors show no seasonality in associated non-native invertebrate animals. *(not relevant since no NIS invertebrates were recorded in bait purchases)*

11. Presentations/Publications/Outreach

Presentations:

Haska, Christina L., Charles Yarish, and Senjie Lin. Assessing the role of bait worm packaging as a potential vector of invasive species to Long Island Sound. Annual Conference of the Phycological Society of America. July, 2008.

Haska, Christina L. Assessing Bait Worm Packaging as a Potential Vector of Invasive Species Introductions into Long Island Sound. Feng Graduate Student Colloquium, Avery Point, CT. May, 2008.

Haska, C., Yarish, C., and Lin, S. 2008. Assessing bait worm packaging as a potential vector of invasive species to Long Island Sound. Presentation at the Long Island Sound Research Conference, New London, CT, October, 2008.

Publications: Manuscripts are currently in preparation.

Outreach: Two workshops have been conducted to begin dissemination of the project results. On November 5, 2008, the results of the study were presented in an open forum (i.e., including interested public) to officials from the Connecticut DEP in Old Lyme. On November 18, a similar presentation was made to the Marine Resources Advisory Council in Setauket, NY. The latter meeting included both officials from the Department of Environmental Conservation and commercial fishermen. An additional presentation will be made to the annual meeting of the New York Lobstermen's Association at the Cornell Cooperative Extension Offices, Riverhead on January 24, 2009 in Riverhead, NY.

Additionally, we have been assisting Nancy Balcom of Connecticut Sea Grant with dissemination of the inserts for the bait-worm boxes that alert fishermen to the potential invasive threat and simple means for minimizing the threat (disposal in land-based garbage containers). This inserts are part of a program funded separately by NOAA called the "Don't Dump Bait Campaign."

12. Other Information: Four undergraduate students participated in the project as Research Assistants, helping with sample acquisition, initial processing, and culturing tasks. The students included Yusuff Abdu and Al Rakiposki (University of Connecticut), and Francisco Cerqueira and Andrew Payne (Purchase College).

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