

Report to Millstone Environmental Laboratory, Ecological Advisory Committee  
Analysis of winter flounder Larvae  
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This is a report of the activities of the second year of a project designed to determine the most likely source population for winter flounder larvae entrained by the Millstone Power Station. Staff scientists at the Environmental Laboratory, Millstone Power Station, Waterford, CT, provided samples. Larvae were collected from the Niantic River, Thames River and an area due west of the Connecticut River, mostly off Westbrook (Figure 1). These areas were known to be, or adjacent to winter flounder nursery locations in Long Island Sound (LIS), and were near the Millstone Station. Larvae samples were staged according to criteria presented in NUSCO (2000). Stage 1 (yolk-sac) & 2 (pre-flexion) winter flounder larvae were collected with a bongo net sampler with a 202- $\mu$ m mesh net at variable depths from February through April 2001. Larvae were sorted on board the sampling vessel and placed in 70% ethanol. A total of 164 Stage 1 & 2 larvae were collected from the Niantic River, 174 from the Thames River area and 198 from the Westbrook area (Table 1).

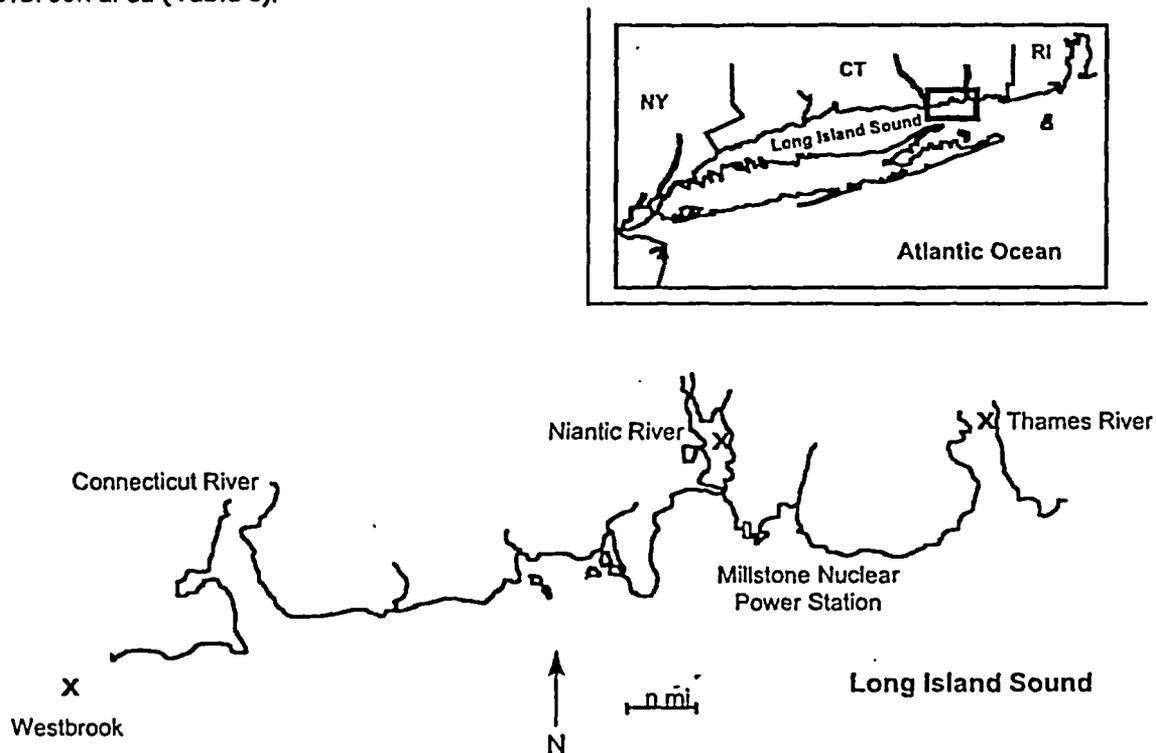


Figure 1. Approximate locations in eastern Long Island Sound (X) where larval winter flounder were collected for genetic stock identification studies during 2001.

Larvae were also collected, using a 333-micron mesh net, from seawater entrained at the Millstone Power Station from March through June 2001. Larval samples collected during this time period were stratified so as to provide an accurate cross-section of larvae entrained by the Station

(i.e., the maximal number of sample were collected during those dates when it was known that the maximal number of larvae are entrained in the Station). A total of 1067 stage 2, stage 3 (flexion) and stage 4 (pre-metamorphosing) entrainment larvae were collected (Table 2).

Table 1. Spawning Stock collection sites and number of larvae processed

Collection site	Date	Number and stage	Total
Niantic River	2.27.01	35 - stage 1	164
		25 - stage 2	
	3.14.01	31 - stage 1	
	3.28.01	19 - stage 2	
		35 - stage 2	
Thames River	3.03.01	12 - stage 1	174
		44 - stage 2	
	3.11.01	19 - stage 1	
	3.18.01	42 - stage 2	
		45 - stage 2	
Westbrook	4.05.01	30 - stage 1	198
		50 - stage 2	
	4.16.01	24 - stage 1	
		34 - stage 2	
	4.26.01	6 - stage 1	
	3 - stage 2		
	51 - stage 3	198	
			536

Table 2. Entrained larvae collection dates & amounts and number of larvae processed

Collection Dates	Number and stage	Total	Collection Dates	Number and stage	Total
3.28.01	14 - stage 2	14	5.14.01	28 - stage 2	104
4.06.01	4 - stage 2	11		66 - stage 3	
	7 - stage 3			10 - stage 4	
4.09.01	45 - stage 2	79	5.21.01	43 - stage 3	63
	34 - stage 3		20 - stage 4		
4.16.01	47 - stage 2	142	5.29.01	41 - stage 3	51
	95 - stage 3			10 - stage 4	
4.23.01	14 - stage 2	190	6.04.01	15 - stage 3	34
	176 - stage 3			19 - stage 4	
4.30.01	18 - stage 2	178	6.11.01	29 - stage 4	29
	160 - stage 3		6.18.01	18 - stage 4	18
5.07.01	29 - stage 2	154			
	104 - stage 3				1067
	21 - stage 4				

Following metamorphosing and settlement, juvenile winter flounder (10-71 mm) were collected on June 22<sup>nd</sup> and September 18<sup>th</sup> of 2000 and July 3<sup>rd</sup> and September 24<sup>th</sup> of 2001 with a 1-meter beam trawl at two locations in the Niantic River (Figure 2; LR and WA). Juveniles were placed in 70% ethanol and then transferred to the lab for analysis. A small piece of muscle tissue was used to isolate genomic DNA from each juvenile (Table 3).

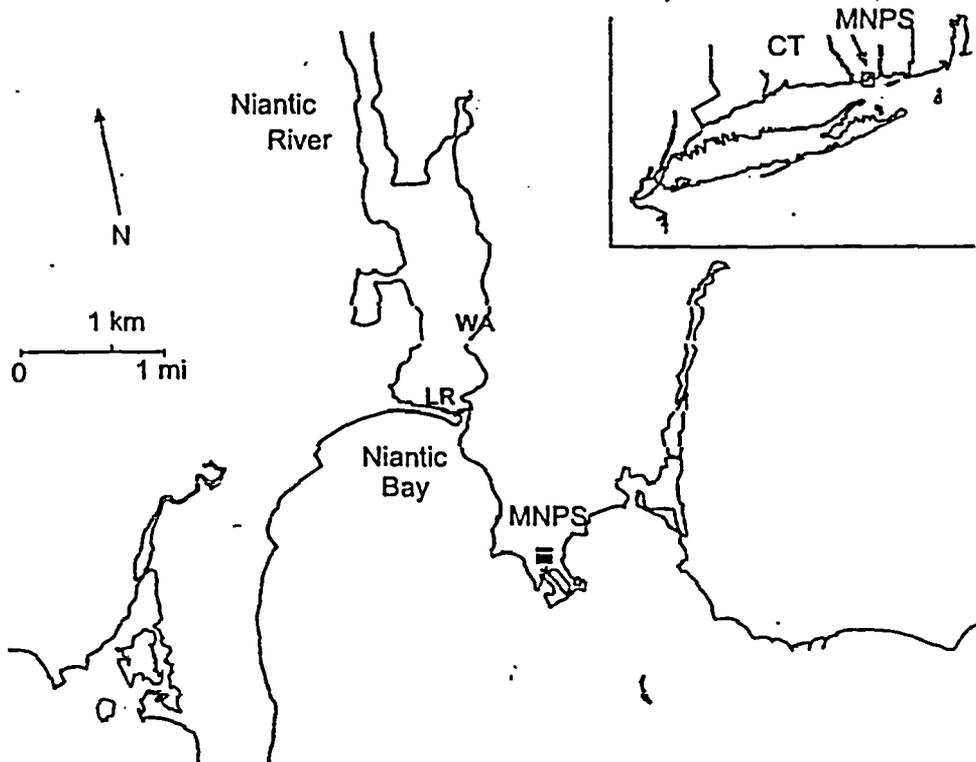


Figure 2. Approximate locations in the Niantic River (LR and WA) where age-0 juvenile winter flounder were collected for genetic stock identification studies during 2000 and 2001.

Table 3. Age-0 juvenile collection dates and number processed

Collection Site (Niantic River)	Date	Number	Collection Site (Niantic River)	Date	Number	Total
LR	6.22.00	57	WA	6.22.00	35	92
	9.18.00	30		9.18.00	35	65
	7.03.01	70		7.03.01	78	148
	9.24.01	45		9.24.01	18	63

### *Genetic analysis*

Genomic DNA was extracted from each sample by the method of Kaplan *et al.* (2001) from each larva and juvenile muscle sample. Genomic DNA was quantified with Pico Green™ (Molecular Probes, Inc.) and comparison to a DNA standard curve. Stage 1 larvae provided 75-100ng of genomic DNA that was sufficient for analysis of 6 microsatellite loci. Stage 2 through 4 larvae and age-0 juveniles gave large amounts of genomic DNA.

To analyze each microsatellite loci, 10ng of genomic DNA was added to a solution containing 10mM Tris, 50mM KCl, 2.5mM MgCl<sub>2</sub>, 0.2mM dNTPs, 0.2μM forward and reverse primers to a final 10μl volume. The forward primer was covalently modified with a D2, D3, or D4 fluorescent tag (Research Genetics Inc. Huntsville, Alabama). The sequence of primers is included in Table 4 as well as the PCR conditions for each primer set. Primer sequences were a kind gift from Susan Douglas and Doug Cook (McGowan & Reith, 1999).

After PCR, the samples were precipitated by addition of 2μl 3M NaAcetate pH 5, 2μl of a 1mg/ml glycogen solution and 50μl of absolute ethanol. The samples were frozen at -70°C for ten minutes and then spun at 30,000xg for 15 minutes. The supernatant was discarded and each sample washed with 75μl of 70% ethanol. The samples were dried and re-suspended in 30μl of formamide that contained a 60-400 bp DNA standard labeled with a D1-fluorescent tag (Beckman Instruments, Pal Alto, CA). The samples were then analyzed on the Beckman Seq-2000™ Capillary Electrophoresis System (frag3 protocol). Microsatellite products were identified by size with an accuracy of 0.25 bp by comparison to standards. Table 4 contains the size ranges and number of alleles for each loci.

### *Statistical analysis*

Statistical analyses of data were performed using PopGene (available as shareware at <http://www.ualberta.ca/~fyeh/>) and the NeuroShell™ Classifier neural net software (Ward Systems Inc, Frederick, MD). PopGene calculated expected heterozygosities as well as an estimate of F<sub>IS</sub> (Cockerham and Weir, 1986). Tests for conformity to Hardy-Weinberg equilibrium were calculated using a Markov chain method. Tests for allele frequency differences were calculated using Fisher's exact test with pair-wise comparison of all samples at all loci that were then combined across loci. Genetic differences were also calculated.

The NeuroShell™ Classifier neural net software was used to assign entrained larvae to likely source locations. This software makes no assumptions about genetic differences among populations and builds an algorithm (i.e., a neural network) that best differentiates differences among the populations. This neural network is then applied to the entrained data. The network is trained on a file (i.e., the training file) that contains the genetic information about larvae collected from the three source areas.

Control experiments to determine the accuracy and resolving power of this approach were carried out in the following manner. A validation experiment was carried out in which the network was trained on one-half of the training sets and then used to classify the other half of each training set. This was repeated 100 times by randomly selecting which samples were included in the training set and which were classified. Through these experiments a mean error rate confidence value was generated. Then the network was trained on the complete training sets (TNN). In a second set of control experiments, the source location of each larva within the training groups was randomized (n=100). The randomized training sets were used to develop networks that were applied to entrained samples (RNN).

After the control experiments were carried out, the TNN was used to classify all entrained larvae and juveniles to the most likely geographical source. The TNN generated a confidence value for the classification of each unknown sample from 0 → 1.0. Samples were assigned to a geographical source population if confidence value exceeded 0.75. A confidence level of 0.75 was chosen for the following reasons: 1) this confidence level would be at least 3 times as great as the next high confidence value and 2) it represents the lowest confidence value with error less than 5% (determined by multiple classification of the same sample). In some cases, the TNN could not assign a sample to a source area with 0.75 confidence but was able to determine that the sample did not belong to a specific source population (less than 0.05 confidence). If the assigned confidence value was the same or similar for all three source populations the sample was assigned to an unknown group. This gave 7 possible groups: Niantic River, Thames River, Westbrook (Connecticut River), not-Niantic River, not-Thames River, not-Westbrook or an unknown location.

### Results:

A major goal of this year's effort was to increase the number of identified alleles, to increase the number of larvae in the source populations and to make the collection of entrained larvae represent with the overall entrainment (i.e., the greatest number of analyzed entrained larvae should come from the date in which the greatest number of larvae are entrained by the plant)

These objectives were met with an increase of 30% in the number of source population larvae (536 in 2001 vs. 423 in 2000). The number of entrained larvae also increased by 3-fold (1067 in 2001 vs. 360 in 2000) and the collection of larvae peaked during late April usually when the greatest numbers of larvae are entrained at the plant, although in 2001, entrained larvae were also abundant in May (DNC, in preparation). The number of identifiable alleles increased from 29 in 2000 to 135 alleles in 2001, thereby increasing the resolving power of the analysis.

Table 4. Primers and PCR conditions, microsatellite sizes, and number of alleles.

Loci	Primer Sequence	Product length (bp)	Annealing T°C	Allele Number
P157	D2-AGTGCAACAACAGATTCCAG(+) GCAGAATGAGTGAAATGTGG(-)	93-195	50°C	40
P159	D3-GTGTGGAGGTCAATGC(+) GGAGCATCATTCATACAC(-)	85-209	53°C	11
A441	D2-CAACTGTGGGTATGTGCCTG(+) GTGTCAGCACTGTGCTTAAACC(-)	89-213	55°C	25
D34	D4-GCCTGGTCTCATTGTGTTCC(+) AGGTAAATGATTCCTGAAGCTG(-)	89-315	55°C	27
I29	D3-GCTTCGGTTACACCTTTGC(+) AGGACAGTGAGGATGTCCG(-)	91-223	55°C	4
J42	D4-CACAACTCAAGATGTTGCG(+) AAGCTCACTGGAAAATAATACCC(-)	95-185	55°C	28

D2, D3 & D4 refer to fluorescent tags on the forward primer.

Individual larvae could have 12 possible products (2 for each primer set) if each locus was heterozygous or one if the locus is homozygous (to a minimum of 6 products). The source populations were examined by POPGENE statistical package. In Table 5, the relevant characteristics of each microsatellite loci are provided. The  $p$  value refers to the likelihood that the loci obey Hardy-Weinberg rules (a  $p$  value  $>0.05$  suggests that it does not). Loci p159 and I29 don't always obey Hardy-Weinberg rules suggesting that they might be inbred but the  $F_{IS}$  values don't suggest that the loci are inbred. The  $Het_{obs}$  values refer to the heterozygosity of the loci among the tested samples. The heterozygosity varied from about 10-80%, which is typical for microsatellite loci. Five out of the 6 loci were very heterozygotic (thereby increasing their resolving power) while one (I29) was not very heterozygotic. In the future, it may be worthwhile to substitute a different marker than the I29 to increase resolving power. It is interesting to note that on the basis of the heterozygosities, the Thames River larvae were less heterozygotic than the Niantic or Westbrook. The Westbrook larvae were the most genetically diverse and may reflect more than one population as these larvae were collected in the open waters of LIS rather than within a specific estuary.

Table 5. Summary statistics for 6 microsatellite loci surveyed in winter flounder.

Population	Microsatellite Loci					
	P157	P159	A441'	D34	I29	J42
<u>Niantic</u>						
P value	0.0000	0.2798	0.0000	0.0000	0.2759	0.0000
Het <sub>obs</sub>	0.7481	0.3926	0.6000	0.7185	0.0667	0.5333
N	148	148	148	148	148	148
$F_{IS}$	0.1485	-0.0515	0.3151	0.1823	-0.0345	0.3597
<u>Thames</u>						
P value	0.0000	0.9817	0.0000	0.0000	0.0000	0.0000
Het <sub>obs</sub>	0.8129	0.0719	0.5971	0.5972	0.1000	0.3669
N	154	154	154	154	154	154
$F_{IS}$	0.0937	-0.0281	0.3263	0.2985	0.1078	0.5927
<u>Westbrook</u>						
P value	0.0000	0.8865	0.0000	0.0000	0.1594	0.0000
Het <sub>obs</sub>	0.7744	0.2359	0.6821	0.4974	0.0103	0.3538
N	195	195	195	195	195	195
$F_{IS}$	-0.0062	-0.0572	0.2133	0.4256	-0.0052	0.5716

$P$  values indicate the probability of conformity to Hardy-Weinberg expectations by the Chi-squared method.  $N$  is the sample size.

The next comparison was to see how distinct the source populations were from each other. This is determined through the  $F_{ST}$  (Fisher's statistic), which is a numerical measurement of the genetic difference, with values greater than 0.05 considered to be significantly genetically distinct and values between 0.025 and 0.05 considered to show less significant but potentially important genetic differences. Table 6 has the results for 2000 & 2001. It is interesting to note that the Niantic River population is distinct from the Thames & Connecticut River area populations in both years. The Thames River larvae are also distinct from the Plum Bank and Westbrook larvae. This

genetic differentiation is geographically linked, i.e., those source populations that most geographically distinct are the most genetically distinct. The other interesting point is that the genetic differences were relatively similar over the last two years, suggesting a temporal stability.

Table 6. The genetic difference between training groups.

Vs.	$F_{ST}$ 2001		$F_{ST}$ 2000	
	Thames	Westbrook	Thames	Plum Bank
Niantic	0.0385	0.0571	0.0384	0.0518
Thames	----	0.0545	----	0.0425
Westbrook		----		----

The NeuroShell neural network classifying program was then trained on the files containing the genetic differences between larvae collected in the Thames, Niantic and Connecticut Rivers. Control experiments were carried out in the following manner. Initially, the neural network was trained on half of the larva from each training area and then used to classify the other half of larva from the same area. Secondly, the order of the samples within the training set(s) was randomized but the correct source location was maintained, Thirdly, both the order and source of the larva in the training set was randomized. These controls demonstrated that this approach had at least 98.5% accuracy in classifying unknown larvae to one of the spawning areas. Samples were assigned to a geographical nursery population if their probability exceeded 0.75. A confidence level of 0.75 was chosen for the following reasons: 1) this confidence level would be at least 3 times as great as the next high confidence value and 2) it represents the lowest confidence value with error less than 5% (determined by multiple classification of the same samples with neural networks). In some cases, the network could not assign a sample to a nursery area but was able to determine that the sample did not belong to a specific nursery population (essentially less than 5% confidence). If the assignment confidence was the same for all 3-nursery populations the sample was assigned to an unknown group. This gave 7 possible groups: Niantic River, Thames River, Westbrook (Connecticut River), not-Niantic River, not-Thames River, not-Westbrook or an unknown location.

It is clear from Table 7 that there were very few larvae that could not be assigned to one of the 1<sup>st</sup> 6 groups (essentially not from any of the tested source areas). The greatest number of entrained larvae came from the Westbrook area (34%) and approximately equal number classified to the other spawning stocks (Niantic River 24%, Thames River 21%). Peak entrainment of Niantic River larvae was in mid-April. Peak entrainment of Thames River larvae was in late May, early June, while peak entrainment of Westbrook larvae occurred in early May at the same time of peak entrainment into the Power Station (Table 7).

Table 7. Classification of entrained larvae to known nursery areas using the trained neural network.  
 Classification (values are expressed as percentage of total)

Collection Date	Niantic River	Thames River	Westbrook	Not Niantic River	Not Thames River	Not Westbrook	Unknown
3.28.01	46.2	7.7	15.4	23.1	0	7.7	0
4.09.01	14.6	29.2	34.8	6.7	7.9	3.4	3.4
4.16.01	29.5	22.7	25.0	6.1	6.1	9.1	1.5
4.23.01	35.6	23.3	21.7	5.0	2.2	11.7	0.6
4.30.01	27.2	15.4	33.3	8.0	9.9	4.3	1.9
5.07.01	16.2	14.0	51.5	9.6	5.9	1.5	1.5
5.14.01	17.2	13.8	46.0	9.2	5.7	4.6	3.4
5.21.01	21.7	30.4	31.9	10.1	0	4.3	1.4
5.28.01	18.8	29.2	29.2	4.2	10.4	2.1	6.3
6.04.01	6.9	24.1	48.3	17.2	3.4	0	0
6.11.01	18.5	18.5	37.0	18.5	7.4	0	0
6.17.01	23.1	38.5	7.7	7.7	7.7	15.4	0
% of all sampled larvae	24.1	21.0	33.5	8.1	5.8	5.7	1.8

Classification to one of the known spawning areas required at least a 0.75 confidence. Classification to the not-spawning area columns required that one of the spawning areas be classified as having <0.05 confidence of being the spawning area for that larvae. The unknown classification was for larvae that had equal confidence to belong to any of the spawning groups.

Juveniles that were collected in 2000 & 2001 from the Niantic River were compared to the tested spawning stocks (Table 8). Once again, very few juveniles could not be assigned to one of the 1<sup>st</sup> six groups. There was no significant difference in the classification of the juveniles collected in early or late summer of both years. LR station, located near the mouth of the river, had initially fewer juveniles classified to the Niantic spawning stock in June 2000 than the WA site that is more upriver. However less difference was seen in September 2000 or in 2001. The great majority of juveniles were classified to the Niantic River and Westbrook areas and not to the Thames River. Also of interest was the size of juveniles assigned to source populations, i.e., were larger juveniles produced within the Niantic River as opposed to smaller fish entering the River from other areas. A one-way analysis of variance indicated no significant difference in the juvenile size by year, month, and station except in September 2000 at the lower river site, where juveniles originating from Westbrook were significantly larger (mean of 43 mm) than those from the Niantic (36 mm) or Thames Rivers (35 mm).

Table 8. Classification of juvenile winter flounder to nursery areas using the trained neural networks.

	Classification (values are expressed as percentage of total)							
	June, 2000	September 2000	June, 2000	September 2000	June, 2001	September 2001	June, 2001	September 2001
	LR	LR	WA	WA	LR	LR	WA	WA
Niantic River	21	23	51	26	20	28	16	17
Thames River	11	10	11	29	24	9	13	11
West Brook	40	37	17	37	27	36	44	33
Not Niantic	5	10	0	0	16	5	16	6
Not Thames	14	7	11	0	4	17	9	17
Not West Brook	7	13	6	6	9	5	2	11
Unknown	2	0	3	3	0	0	0	6

#### Conclusions & Discussion:

Previous work suggests that there are discrete breeding stocks of winter flounder, based on morphometric, meristic, tagging studies and other factors (Pearcy 1962a, 1962b, Berry et al., 1965). Work over the past few decades by the Connecticut Department of Environmental Protection has suggested that distinct spawning and nursery areas for winter flounder exist within LIS (Howell et al., 1999). One such nursery area is in the Niantic River that is nearby to the Millstone Power Station. In general, marine fish show less genetic differentiation than freshwater or anadromous fishes since marine environments are less fragmented than freshwater environments (Carvalho, 1994; Ward et al., 1994). Marine organisms with a planktonic phase have a high potential for physically and biologically mediated dispersal. Nonetheless, evidence does suggest that larval retention (Jordan et al., 2000), cohort fidelity (Sinclair 1988), geographical structures and impediments (Ruzzante et al., 1998) and natal homing instincts (Nielsen et al., 1999) may limit gene flow. Marine species such as cod, hake, herring and squid have previously shown little genetic population differentiation by allozyme markers and thought to be homogeneous over large geographical ranges. Recent examination with microsatellite loci has revealed fine levels of population structure (Bentzen et al., 1996; O'Connell et al., 1998, Lundy et al., 1999, Shaw et al., 1999).

Microsatellite loci form a class of highly polymorphic and informative regions of chromosomal DNA that have found great usage for studies of intra-specific population structures, as well as hybridization events, linkage mapping, paternity testing and pedigree analysis (Hughes & Queller et al., 1993, Roy et. al., 1994, Dowling et. al., 1997). Although statistically significant genetic differences do not always have biological significance (Waples 1998) they can play an

important role in fishery management issues or in instances of efforts to recover commercial fishing industries.

During normal Station operations, cooling water withdrawn by the Station causes the entrainment and death of millions of winter flounder larvae. The issue of plant impact due to winter flounder larval entrainment has been addressed by population dynamic modeling (Lorda et al., 2000). This modeling has focused on the Niantic River winter flounder stock and among the information required is an estimate of the annual reproductive output removed by entrainment. This fraction was determined using an indirect method (the mass-balance model). However, the present genetic analysis provides a more direct quantitative estimate of entrainment loss by source population. Thus, larvae in early developmental stages were collected from areas known to be near spawning grounds. The young stage 1 & 2 larvae appear to have limited geographical dispersal and hence likely retain genetic differences. Comparison of genetic differences between these groups demonstrates a relatively high degree of difference (Nei's genetic difference  $>0.05$ ). This genetic differentiation is geographically based with the greatest difference seen between the Westbrook and the Thames River source areas that are separated by 20.5 miles. Though only separated by 5 miles, the Thames and Niantic River source areas had relatively substantial genetic separation between them (Nei's genetic difference = 0.036), suggesting that one or more factors might be limiting gene flow. The same genetic differences were seen in larvae collected in 2000, suggesting a temporal stability to these genetic differences.

There is no apparent geographical or physical barrier to gene flow between these areas so natal homing instincts or selective pressures may be responsible. These differences were sufficient to provide the resolution to assign entrained larvae or older, settled juveniles to the tested populations. Many different approaches have been used to assign individuals of unknown origin to populations based on the genetic distance between individuals and populations (e.g., neighbor-joining trees, Estoup et al., 1998, likelihood of the multi-locus genotype and Bayesian, Cornuet et al., 1999). A key component of any of these approaches is that they have the ability to correctly assign individuals, but a common drawback is that if the origin of the individual is not represented in the reference populations, most methods will still designate a wrong population of origin (Cornuet et al., 1999). Many approaches are based on two explicit assumptions that all loci are at Hardy-Weinberg equilibrium and at linkage equilibrium. Other constraints are the levels of differentiation between tested populations; the ability to sample all, or virtually all of the potentially contributing stocks, the temporal stability of the microsatellite markers and a large sample size that contains representation for all donor populations (Letcher and King, 1999; Smouse et al., 1990). Newer approaches have attempted to overcome these limitations (e.g., Bayesian) and maximizing the accuracy of assignment depends in part on the  $F_{ST}$  values, population sizes and loci number. The large differences in  $F_{ST}$  values among tested reference populations in this work, coupled with large population sizes (on average 150 individuals) and loci with large numbers of alleles (135) should allow for the maximal accuracy in assignment to reference populations. It is clear that we have not sampled from all of the possible donor stocks in LIS or surrounding areas, but focusing on a smaller geographical range across which all stocks can be sampled adequately, minimizes this problem.

Recently, several investigators have begun to use non-supervised or artificial neural networks (ANN or TNN) to assign individuals to populations based on genetic differences (Brosse et al., 1999; 2001; Wu-Catherine 2000). These neural networks are trained on populations with known genetic differences and then applied to unknown individuals. Neural networks make no prior assumptions about the characteristics of the training sets and develop algorithms that maximize its

ability to correctly identify unknown individuals to populations. These ANN have been used in a wide range of areas including assessments of fish abundance and spatial occupancies (Brosse et. al., 1999).

The TNN used in this work was trained on microsatellite data generated from the populations found in the Niantic, Thames and Westbrook areas. To test the accuracy of this approach for correct assignment of individuals to populations, confidence values were generated and classification was compared to neural networks trained on randomized (and incorrect) training sets (i.e., the RNNs). The RNNs lost all ability to classify individuals and classification was essentially random. The TNNs classified individuals with high accuracy. A confidence value below 0.75 was the lowest confidence used to assign an individual to a source population. Even when the TNN could not assign an individual to a specific population with at least 0.75 confidence, it was capable of determining that an individual was not from a specific population (<0.05 confidence). When the TNN was applied to the entrained larvae and collected juveniles, the assignments, namely that not all of the entrained larvae were from the Niantic River stock, are in agreement with other approaches (e.g., mass-balance model based on larval dispersal). Previous mass-balance approaches (NUSCO, 2000) indicated that during 1984-98, about 12% to 59% of the entrained larvae were from the Niantic River with a long term average of 25.4% that is in agreement with the results seen here (24.1%). Previous work has also suggested that larval entrainment from the Niantic River area is highest earlier in spring. This likely occurs because this area is closest to the Station and larvae displaced from the River make up a greater proportion of all larvae found in Niantic Bay. As the season progresses more larvae from other sources are transported by tidal currents into this area and locally produced larvae are less dominant. Large assignments were made to the tested population that lies 15 miles to the west near the Connecticut River. The Thames River, which is 5 miles to the east, contributed far fewer larvae. This suggests that currents and tidal flow transport winter flounder larvae in April and May predominantly from a west-to-east direction. This is supported by the fact that the contribution of the Thames River population is also highest June.

The assignment of larvae to the Westbrook (i.e., Connecticut River) area reaches a peak in mid-May, when the contribution of larvae from the Niantic River has reached a nadir. The peak entrainment in the Station occurs in late April & early May when the contribution from the Westbrook area is greatest.

Over 80% of the larvae could be definitively assigned to one of the tested populations with at least a 0.75 confidence value. Of the remaining 20%, the TNN could determine that they did not belong to one of the populations and the confidence values were roughly divided between the remaining two. There were very few larvae (2%) that could not be assigned to the tested populations or were known not to have come from a specific population.

This approach is also able to classify the most likely source population of young-of-the-year juvenile flounder. Though only the genetic differences between larvae collected in 2001 were used in the TNN, we had previously characterized larvae in 2000 and found the genetic differences between the source populations to be almost identical as they were in 2001. Juvenile flounder of the 2000 & 2001 year class that were collected in July and September of each year from two sites in the Niantic River were then assigned to the tested populations with the TNN. The lower river site had initially a smaller proportion of juveniles assigned to the Niantic River than the mid-river site, where there were more juveniles resulting from the Thames River. Also, there were substantial numbers of juvenile flounder from the Westbrook area that were captured in the Niantic River. Overall, in 2001, 21% of the juveniles were classified as of Niantic River origin, 15% from the Thames River, 25% from the Westbrook area and the source of 28% of the juveniles could not be

determined. These results clearly demonstrate that though the stage 1 larvae found in up-river spawning areas have substantial genetic differences with larvae from other spawning areas, the young-of-the-year that settle in the Niantic River are from a wide geographic area.

In terms of fisheries management, this approach allows for the estimation of the impact of a commercial operation (i.e., the Power Station) on specific flounder spawning groups and also allows for the direct linkage between a flounder spawning stock and recruitment to juveniles. Both of these factors are critical components of any fishery management plan that attempts to maintain a commercially viable winter flounder population in LIS.

#### Future experiments:

- If these experiments were continued for another year, I would recommend that we do so at the same level of effort as this year. Collection for an additional year will allow us to determine the variance in the genetic difference between the spawning populations as well as the variance in the entrainment of larvae. If this information is correlated with hydrodynamic information (tides, currents, spring thaws, etc.) and applied to current plant operations, then it might be possible to predict the entrainment from spawning areas in future years with a high level of confidence.
- With information from a third year, it will be possible to determine  $N_e$ , the effective adult spawning population in the different river areas.  $N_e$  refers to the amount of adults that contribute the genes to 95% of the population and is a useful value for fishery management issues and identifies the number of females producing the larvae found in each river system.

#### References:

Bentzen, P., C.T., Taggart, D.E., Ruzzante and D. Cook. 1996. Microsatellite polymorphism and the population structure of Atlantic cod (*Gadus morhua*) in the Northwest Atlantic. *Can. J. Fish. Aquat. Sci.*, 53:2706-2721.

Berry, R., S. Salia, S. and D. Horton. 1965. Growth studies of winter flounder, *Pseudopleuronectes americanus* (Walbaum) in Rhode Island. *Trans. Am. Fish. Soc.* 94:259-362.

Brosse, S., J.F., Guegan, J.N., Tourenq and S. Lek. 1999. The use of neural networks to assess fish abundance and spatial occupancy in the littoral zone of a mesotrophic lake. *Ecol. Model.* 120:299-311.

Brosse, S. J.L. Giraudel and S. Lek. 2001. Utilization of non-supervised neural networks and principal component analysis to study fish assemblages. *Ecol. Model.* 146:159-166.

Carvalho, G.R. and L. Hauser. 1994. Molecular genetics and the stock concept in fisheries. *Rev. Fish Biol. Fish.* 4:326-350,

Cockerham, C.C. and B.S. Weir. 1986. Estimation of inbreeding parameters in stratified populations. *Ann. Hum. Genet.* 50:271-81.

Cornuet, J.M. Piry, S., Luikart, G., Estoup, A. and M. Solignac. 1999. New methods employing multilocus genotypes to select or exclude populations as origins of individuals. *Genetics*, 153:1989-2000.

DNC 2002. In preparation: Winter Flounder Studies. Monitoring the marine environment of Long Island Sound at Millstone Nuclear Power Station, Waterford, CT. Annual Report 2001. Dominion Nuclear Connecticut Inc.