March 18, 2004

Mr. Glenn Haas, Director
Division of Watershed Management
Department of Environmental Protection
1 Winter Street
Boston, MA 02108

Ms. Linda Murphy, Director
Office of Ecosystem Protection
U.S. Environmental Protection Agency
Water Technical Unit "SEW"
P.O. Box 8127
Boston, MA 02114

Re: Massachusetts Water Resources Authority, Permit Number MA0103284
Part I.7. Ambient Monitoring Plan

Dear Mr. Haas and Ms. Murphy:

Attached for your review is a description of the special study of flounder skin lesions MWRA is proposing to carry out in April, 2004, as requested by the U.S. Environmental Protection Agency and the Massachusetts Department of Environmental Protection. Please contact me at 617-788-4708 if you have any questions about this study.

Sincerely,

Andrea C. Rex, Ph. D.
Director, Environmental Quality Department

Attachment: Design for MWRA Flounder Skin Ulcer Special Study, 2004

Cc:

Environmental Protection Agency, Region I (EPA)
Matthew Liebman
Janet Labonte-Deshais
Eric Hall
Roger Janson

Massachusetts Department of Environmental Protection (DEP)
Cathy Coniaris

Outfall Monitoring Science Advisory Panel
Andrew Solow
Robert Beardsley
Norb Jaworski
Robert Kenney
Scott Nixon
Judy Pederson
Michael Shiaris
James Shine
Juanita Urban-Rich

Massachusetts Division of Marine Fisheries
Paul Diodati
Jack Schwartz
David Pierce

National Marine Fisheries Service
Chris Mantzaris

Stellwagen Bank National Marine Sanctuary
Craig MacDonald

EOEA
Karl Honkonen

Hyannis Library
Ann-Louise Harries

MWRA Library
Mary Lydon

Cape Cod Commission
Steve Tucker

Design for MWRA Flounder Skin Ulcer Special Study, 2004

Principal Investigator, Michael J. Moore, WHOI

Task: Extended spatial survey and diagnostic analysis of blind side ulcers.

Rationale: In the April 2003 flounder survey, a novel finding of a significant prevalence of blind side ulcerative dermatitis was recorded (Moore 2003), with the highest prevalence in Western Massachusetts Bay. The condition was absent from Eastern Cape Cod Bay. No etiological agent was identified despite microbiological and histopathological investigations. Review of the data by the Outfall Monitoring Science Advisory Panel on October 21, 2003 recommended further study of this condition. A meeting was held at MA DMF in Gloucester MA on November 18, 2003 to plan these studies. As a result of that meeting, MWRA agreed to prepare a field guide to external lesions in winter flounder to better categorize each entity, and to design and carry out a sampling study that would extend the spatial coverage of the survey and further investigate the possible etiology of these ulcers.

Study Design

Stations: In addition to the four core flounder monitoring stations shown in Figure 1 (Deer Island Flats, Nantasket Beach, Outfall Site and Eastern Cape Cod Bay), the former flounder monitoring station in Broad Sound will be occupied as part of this special study. Liver samples from fifty fish from each station will be collected for histopathology analyses as detailed in Lefkovitz *et al*. (2002). Filet and liver samples will be collected from 15 fish from each site, and archived for possible contaminant analyses following procedures detailed in Lefkovitz *et al*. 2002).

In addition to those five sites, we will also occupy four new stations designed to be north-east (2) and south (2) of the outfall site as shown in Figure 1. Precise location of these new stations will be dependent on the availability of winter flounder and trawlable bottom selected in consultation with the vessel management, and in consultation with Battelle and MWRA staff to maximize integration with other data sets such as the sediment *Clostridium* spores. We will attempt to coincide with the *Clostridium* spore stations shown in Figure 1, taking care not to disturb the exact benthic sampling location with the trawl equipment.

External lesion evaluations: At all nine stations 50 flounder larger than 300 mm in total length will be measured, scales removed for age analysis, and external condition assessed using the classification shown in Figure 2, using definitions of external lesions from Sindermann *et al*. (1976). Data will be recorded on the data sheet shown in Table 1. Any ulcer found will be sampled for microbiological culture as soon as it is observed, by using a sterile loop to inoculate culture media that will be held on ice for return to the laboratory of Dr Roxanna Smolowitz at the Marine Biological Laboratory in Woods Hole MA. Additionally the flounder carcasses bearing ulcers will also be bagged, tagged and placed on ice for delivery within 18 hours to Woods Hole for further microbiological and histopathological analysis in an attempt to diagnose the etiological agent(s) involved in these lesions. The evaluations to be conducted by Dr Smolowitz are described in Attachment A.

Briefly, those evaluations will seek to determine whether bacteria (aerobic or anaerobic), funguses, or viruses are associated with the blind-side ulcers. Bacterial isolates cultured from the ulcers will be screened to attempt to identify specific strains that may be associated with the ulcers.

The study design assumes that a total of 35 ulcerated fish will be submitted to Dr Smolowitz. This number was determined based on the varying prevalence encountered at the different stations surveyed in 2003.

Reporting: Field observations on the presence, prevalence, and severity of winter flounder blind-side ulcers will be reported to regulators and OMSAP within 30 days of survey completion.

Results from the microbiological and histopathological evaluations should be available by early July. A summary report will be prepared combining those results with the field observations. This report should be available by mid-august; mwra will notify regulators in the event circumstances beyond the control of MWRA or the researchers prevent submission of the summary report by August 31.

It is important to note from the outset, that there can not be a major expectation of these studies revealing the etiological agent(s) of the condition, unless specific known ulcerogenic pathogens are recognized. As discussed previously (Moore 2003), past studies have shown these conditions to often reflect a combined impact of altered water quality, other stressors and infectious agents. Therefore data to be collected under this task will have to be interpreted in the light of all available information about water and sediment quality in the region.

References

Lefkovitz, L., Abramson, S., and Moore, M. 2002. Combined Work/Quality Assurance Project Plan (CW/QAPP) for Fish and Shellfish Monitoring: 2002/2005. Boston: Massachusetts Water Resources Authority. Report ENQUAD ms-078. 71 p.

Moore , M. J. 2003. Winter flounder ulcer final report for fish and shellfish monitoring Task 29, Task Order 11, MWRA Harbor and Outfall Monitoring Project. Woods Hole Oceanographic Institution.

Sindermann, C. J., Ziskowski, J., and Anderson, V. T. 1976. A guide for the recognition of some disease conditions and abnormalities in marine fish. Sandy Hook Laboratory, Northeast Fisheries center, National Marine Fisheries Service, NOAA, Highlands, NJ 07732. Technical Series Report No. 14. 60

Figure 1. The four routine flounder stations (DI, OS, NB, and ECCB), the Broad Sound site(BS), and 4 additional stations will be surveyed for ulcer prevalence. The four additional stations have been chosen to be alongside selected MWRA benthic monitoring stations (sediment *Clostridium perfringens* data shown here).

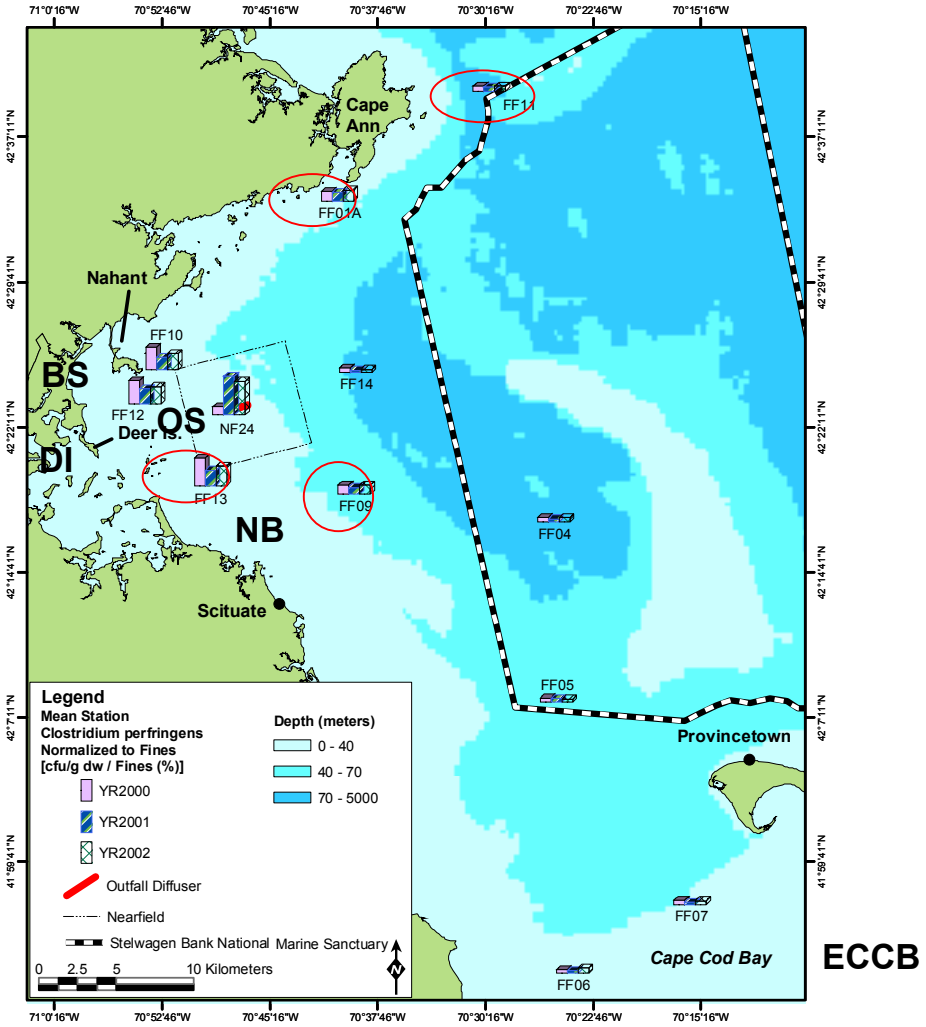
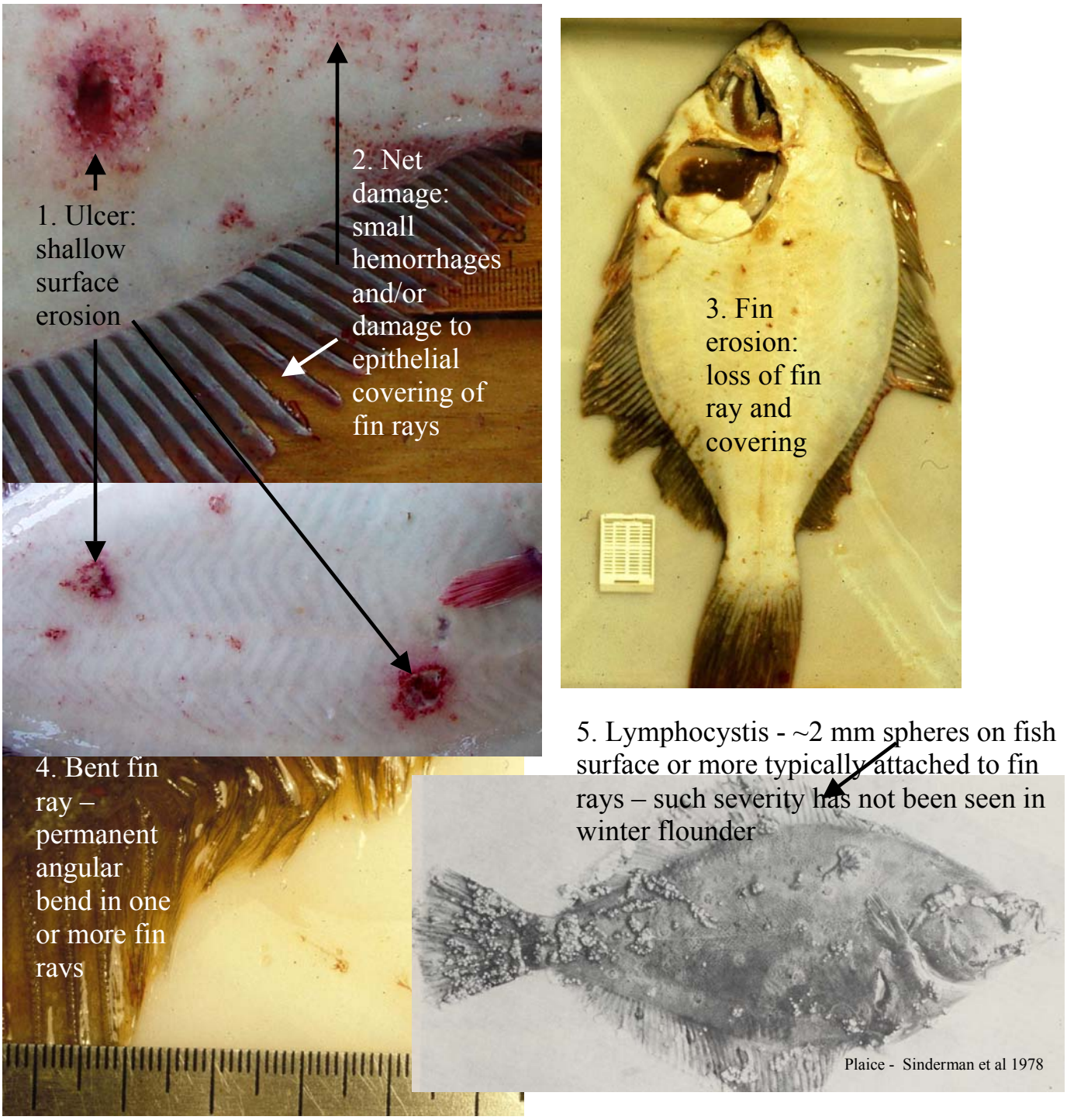


Figure 2 - Scoring External Lesions On Winter Flounder



Grade the severity of the lesions present. The severity grade should be an aggregate estimate of how severely each fish is affected overall with a particular lesion type. Lesion severity should be estimated on a range of 0: absent, 1: mild, 2: moderate, 3: severe and 4; extreme. Record date, time and latitude and longitude of sample. The ulcer, fin erosion and lymphocystis cases would be a severity 4

Table 1. Score sheet for External Lesions on Winter Flounder – The usual station log and fish morphology forms (Lefkovitz *et al.* 2002) will also be used.

Winter flounder external lesion record. Grade each fish for overall lesions severity for each lesion type

Date	Station #/ ID	Fish #	Ulcer	Net damage	Fin erosion	Bent fin ray	Lymphocystis	Other

ATTACHMENT A

Laboratory evaluations of flounder ulcers

Principal Investigator, Roxanna Smolowitz, DVM MBL

Objectives:

We will evaluate a maximum of 5 animals from each of 9 sites as determined by Dr. Moore and the MWRA. Dr. Moore will be responsible for initial evaluation of the ulcerative lesions, microbial samping and selection of fish to be examined in the laboratory.

Field Processing Methods

One ulcer from each selected fish will be sampled for microbial analysis using both aerobic and anaerobic methods. Specifically, for aerobic methods, a lesion will be flushed with sterile sea water then sampled using a sterile bacterial loop. Content of the loop will be spread, in a quadrant fashion using standard microbial techniques, on an culture plate containing Marine Brain\Heart Infusion Agar (MBHIA). The plate will be secured with parafilm, identified, placed in a plastic ziplock bag and held at a cool temperature till arrival at the lab the following morning. For anaerobic samples, the sample lesion will be sampled a second time but this time the sample will be used to innoculate a labeled sterile Bacto Anaerobic Agar plate. The plate will be secured with parafilm and will be inserted into a GasPak Pouch (BBL). Within twenty minutes of sampling the GasPak Pouch will then be evacuated of air, sealed using a sealing bar, then the liquid-activating agent (supplied with the pouch) will be inserted in the compartment provided within the pouch to create an anaerobic atmosphere. The sampled ulcer from each fish will be identified in writing by Dr. Moore. After internal sampling of each fish to be conducted on board by Dr. Moore, the animal will be identified, placed in a plastic bag and held on ice till delivery at the laboratory.

Microbiological Evaluations

At the laboratory and at the beginning of the necropsy process, a second microbial sampling will be repeated on the other half of the lesions from two of the five fish from each sample site. This will be done in order to compare onboard and laboratory sampling results and to ensure retrieval of bacteria from the lesions. Additionally, one ulcer from two of five fish from each site will be cultured in a similar manner for fungus on PDA media (any fungus isolated will be held, and not submitted for identification, till histology verifies that fungus was involved in destruction of the tissues)

All plates (aerobic and anaerobic, and collected both on board and in the laboratory) will be held at room temperature for 2-5 days. Specific colonies (based on different colony morphologies as identified on the culture plates) will be sampled and isolated on additional culture plates. Once pure cultures are obtained (by repeatedly touch point colony sampling and streaking new plates), they will be sent to Microtechnology, Inc. for identification. Only those bacteria that appear to be common to the ulcers from the fish at each site will be identified. Bacteria that are isolated from only a few of the fish at each site will not be further isolated or identified.

Gross and Microscopic Evaluation of Fish

Animals will be necropsied upon the day of arrival at the Marine Biological Laboratory. Each animal will be removed from the plastic bag, identified with the number provided by Dr. Moore, and will be given a new unique necropsy number at the laboratory. The external surface of the animal will be evaluated grossly and all ulcers will be described and digitally photographed. A small portion of an ulcer edge will be removed from each fish and frozen at -80 °C for potential viral screening (depending on histological findings). A second small piece of an ulcer- edge will be placed in a TEM fixative for potential TEM evaluation (depending on histological evaluation of the lesions and additional funding by the MWRA). The internal organs of the animals will be examined at necropsy. Histological samples will be removed from ulcers (and other external lesions as appropriate), liver, kidney, spleen and gills, and other organs, if abnormal, and will be fixed in 10% buffered formalin. After fixation, the tissues will be trimmed, placed in cassettes and set to Histological Services, Inc. (Rhode Island) for processing into hematoxylin and eosin stained microscopic slides. These slides will be returned to the MBL.

At the MBL, the histological slides will be evaluated by Dr. Smolowitz The ulcer sections will be evaluated for depth of penetration into the tissues, degree of inflammation and tissue destruction, and for potential traumatic or infectious agents including bacteria, fungus, virus. Paraffin embedded tissues from which the H&E slides were made, will be sent back to Histological Services or MIT Histology Services for production of specially stained sections. Special stains that may be requested are: tissue gram (that highlight bacteria in sections), GMS (that highlight fungal elements in sections) and Feulgen stains (that highlight DNA bacteria in sections). Special stains to be requested will be determined based on the initial histological evaluation of the H&E stained sections from the lesions.

All histological findings will be documented and when appropriate, digital photos will be provided to illustrate findings.

Reporting of Findings

A final report will be generated that will include selected gross and microscopic photos of the fish ulcers, description of the gross and microscopic findings and microbial findings. All paraffin embedded tissues and tissues for viral and TEM evaluation will be archived at the MBL.

If warranted and if separate funding is provided by MWRA, further work on the viral and TEM samples will be conducted.