Effects of Water Movement on the Fluctuation of Oxygen Concentration in the Lower Layer of Gokasho Bay on the East Coast of Honshu Island, Japan

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ABSTRACT

The physical factors affecting the fluctuations of oxygen-deficient water masses in the lower layer (below the pycnocline) of Gokasho Bay were investigated. In this area, sea bream, yellowtail and pearl oysters are cultivated. An oxygen budget was estimated using a two-layered box model analysis with the results of field observations. The effects of water movements on the fluctuation of dissolved oxygen (DO) concentration were then examined. Oxygen-deficient water masses existed in the lower layer throughout the stratified season (from May to October). The factors which caused the oxygen depletion were clarified by the results of an oxygen budget analysis. In summer, oxygen depletion was caused by low vertical diffusion. In early autumn, a very high consumption rate of DO caused oxygen depletion, despite high vertical diffusion and high horizontal advection. Water masses from outside the bay were found to intrude into the bay intermittently. DO concentrations showed short periodic fluctuations due to these intrusions. The intrusions were caused by coastal upwelling and concomitant ascent of the pycnocline outside the bay. Because DO concentrations outside the bay were higher than those in the lower layer of the bay during the stratified season, the intrusions increased the supply of DO in the bay. The oxygen-deficient water mass disappeared temporarily after the intrusion. Water movement was the significant factor which supplied DO in the bay during the stratified season.

INTRODUCTION

Many workers have described the spatial and temporal distribution of the oxygen-deficient water masses in the coastal area of Japan (e.g., Ochi and Takeoka 1986, Joh 1989, Sasaki 1989). To clarify the processes of oxygen depletion, it is important to study the physical, chemical and biological factors that control water quality in the bay. Munekage et al. (1991) observed the effects of the intrusion of oceanic water on oxygen depletion and Unoki et al. (1985) considered water movements in his study of oxygen consumption rates. Water movements and their effects on dissolved oxygen (DO) concentrations differ among the bays; the physical characteristics of each bay must therefore be examined to elucidate the mechanism of oxygen depletion. In this paper, we examined the physical factors affecting oxygen depletion in Gokasho Bay. Coastal water masses outside the bay intrude into the bay intermittently (Abo et al. 1996). We conclude that such intermittent events of water movements are important to the fluctuations of oxygen-deficient water masses.

OBSERVATIONS

Gokasho Bay is located on the southern coast of central Japan (Fig. 1). Sea bream, yellowtail and pearl oysters are cultivated in this bay, and fish farms are concentrated in one of the inlets of the bay (stn. 6-8).

Fig. 1. Map of Gokasho Bay and the observation stations. Heavily stippled areas denote the distribution of fish farms.
Vertical profiles of water temperature, salinity and DO were measured at weekly (stn. 4-12) and at monthly (stn. 1-12) intervals in 1992 using an STD and DO meter. Short periodic measurements (every 30 min) were also taken in 1991 and 1994: water temperature, salinity and current velocity at depths of 3 m, 9 m and 15 m below sea level at stn. 8 in September and October of 1991, and water temperature and DO at the depth of 1 m above the seabed at stn. 8 in August and September of 1994.

RESULTS AND DISCUSSION

OXYGEN DEPLETION

Oxygen depletion occurred in the stratified season. Fig. 2 shows the vertical profile of DO concentration in the bay. Oxygen-deficient water masses were formed in the lower layer of the bay. At stn. 7 (near the fish farms), DO concentrations were less than 1 ppm in the bottom layer. The influences of aquaculture activities on oxygen depletion were estimated by comparing fluctuations of DO concentration at stn. 7 with those at stn. 12 (about 1 km from the fish farms) from April to October of 1992 (Fig. 3). The two stations have almost the same water depth and are equal distance from the mouth of the bay. Although there were no differences in the fluctuations of density (water temperature and salinity) between the two stations, DO concentrations at stn. 7 were lower than those at stn. 12. At stn. 7, oxygen-deficient water masses (less than 3 ppm) appeared in the stratified season (from May to October) and the concentration was occasionally less than 1 ppm at the bottom. On the other hand, at stn. 12, oxygen-deficient water masses only appeared intermittently from June to August, and DO concentration was never less than 1 ppm. These results suggest that aquaculture activity, that is, loading of organic material from the aquaculture area, caused the oxygen depletion.

BUDGET OF DO

The oxygen budget in the lower layer of the bay was estimated by using the two-layered box model analysis with the results of field observations. For this model, we defined the inlet as inside the box (stn. 6-11), defined the adjacent sea area (sm. 4-5), and separated the box into upper and lower layers at a depth of 8.5 m below sea level. We defined vertical diffusion (Dij) and advection (Qij and Qij'), and disregarded other diffusion and advection (Fig. 4).

Vertical diffusion and advection were estimated by considering the conservation of water mass and salinity. The budgets of water mass and salinity are expressed as follows.

\[
R + Q_{21} - Q_{13} = 0
\]

\[
Q_{42} - Q_{24} = 0
\]

\[
V_1 \frac{dC_1}{dt} = -(C_2 - C_1)D_{12} + C_2 Q_{21} - C_1 Q_{13}
\]

\[
V_2 \frac{dC_2}{dt} = -(C_1 - C_2)D_{12} - C_2 Q_{21} + C_1 Q_{13}
\]

Where \( C_n \) is the salinity in box-n (n=1, 2, 3, 4), \( V_n \) is the volume of box-n, R is the freshwater inflow into box-1, \( Q_{in} \) is the flux of water from box-n to box-m (advection) and \( D_{ij} \) is the vertical diffusion. If the salinity \( C_n \) is observed at intervals of dt, the advection and diffusion can be estimated. Using these diffusion and advection estimates, the oxygen budget can be calculated. The oxygen budget in the lower layer (box-2) is as follows.
\[ V_2 \frac{dC_2}{dt} = (C_{12} - C_{21})D_{21} + C_{2}Q_{21} + C_{2}Q_{22} - P \]

Here, \( C_n \) is the DO concentration in box-\( n \) and \( P \) is the consumption of DO. This consumption of DO consists of the consumption by water and bottom sediment and respiration of fish. Production of DO by photosynthesis is negligible in the lower layer of the bay.

Fig. 5a shows the vertical diffusion (\( D_{21} \)) and the horizontal advection (\( Q_{21} \)) estimated by the box model analysis from April to October in 1992. The vertical diffusion was low in July and August and high in September and October. The horizontal advection showed short periodic fluctuations from April to August and got higher in September and October. In the stratified season, the vertical diffusion was low. In autumn, both the vertical diffusion and horizontal advection were high. Fig. 5b shows the consumption rates of DO in the lower layer of the bay; the consumption rate was high in September and October.

The results of the oxygen budget clarified the factors affecting oxygen depletion. The fluctuations of DO concentration at stn. 7 were correlated with vertical diffusion, horizontal advection and consumption rates of DO. In summer, oxygen depletion was caused by low vertical diffusion due to the stratification. In autumn, a very high consumption rate of DO caused oxygen depletion, despite high vertical and horizontal fluxes of oxygen.

The horizontal advection and consumption rate of DO also showed short periodic fluctuations distinct from the seasonal fluctuation. Low DO concentration layer recovered intermittently relative to the short periodic fluctuations of horizontal advection and consumption rates of DO. These short periodic fluctuations were related to the intermittent events of water movements, discussed in the following sections.

**WATER MOVEMENT**

Distinctive water movements were found in Gokasho Bay, that is, cold and high-saline water masses intruded into the bay intermittently. Fig. 6 shows the time series of current velocity, water temperature and salinity at stn. 8. Arrows in the figure denote the times when cold and high-saline water masses outside the bay intruded into the lower layer of the bay.

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**Fig. 4.** Schematic view of two-layered box model analysis.

**Fig. 5.** (a) Seasonal variations of horizontal advection and vertical diffusion as estimated by the two-layered box model analysis. (b) Seasonal variations in the consumption rate of dissolved oxygen.

**Fig. 6.** Time series of current velocity at depths of 3 m, 9 m and 15 m at stn. 8. Water temperature and salinity at depth of 15 m below sea level at stn. 8. Positive values of current velocity denote the inflow. Arrows denote intrusion events.
These intrusions occur as a consequence of the coastal upwelling induced by alongshore wind (Abo et al. 1996). In alongshore (westerly) winds, the Coriolis force causes the surface water outside the bay to move away from the shore (Ekman transport). The surface water moving away from the shore is replaced by deeper water that upwells close to shore, and the thermocline outside the bay ascends (coastal upwelling). With intermittent winds, the thermocline outside the bay fluctuates vertically, responding to the wind. When the ascent of the thermocline outside the bay reaches a shallower layer than the depth of the mouth of the bay, water masses below the thermocline are able to intrude into the bay.

**EFFECTS OF WATER MOVEMENT ON DO**

DO concentrations showed short periodic fluctuations. The low oxygen water masses in the lower layer disappeared intermittently (Fig. 3a). These intermittent renewals of low oxygen water masses were caused by the short periodic water movements, that is, by the intermittent intrusions of coastal water masses from outside the bay.

Since DO concentrations outside the bay were higher than those in the lower layer of the bay in the stratified season, the intrusions added a supply of DO into the lower layer of the bay. Fig. 7 shows the supply of DO due to the intrusions of cold and high-saline water masses outside the bay. On August 31, water temperature and salinity in the bay were higher than 23.4°C and lower than 33.8 psu (practical salinity unit), respectively. There was a low oxygen water mass (less than 1 ppm) in the lower layer of the bay. On September 7, a water mass having low temperature, high salinity, and high DO concentration (more than 4 ppm) intruded into the lower layer of the bay. DO was renewed due to this intrusion.

Such intrusions and subsequent renewals of DO occurred intermittently. Fig. 8 shows the sudden recoveries of DO in the lower layer of the bay (stn. 8) due to the intrusions of cold water masses from outside the bay. As a result, oxygen-deficient water masses disappeared temporarily after the intrusions. It is concluded that such intrusions were significant factors in the renewal of oxygen in the lower layer of Gokasho Bay.

**SUMMARY**

Oxygen-deficient water masses were formed in the lower layer of Gokasho Bay in the stratified season. The results of the DO budget suggest that summer oxygen depletion is caused by low vertical mixing, due to a strong stratification; whereas, in autumn, a very high consumption rate of DO caused oxygen depletion despite a higher horizontal advection.

Water masses below the pycnocline outside the bay, which have high density and high DO concentration, in-
truded into the bay. The intrusions induced a flux of DO into the lower layer of the bay; low oxygen concentrations disappeared temporarily after the intrusion. The intrusions were significant factors in supplying oxygen to the lower layer of the bay. Thus, the effects of water movements must be considered when studying oxygen depletion in coastal areas such as Gokasho Bay.

LITERATURE CITED


Concepts of Herd Health for Shrimp

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ABSTRACT

Identifying a risk factor as a cause requires the demonstration of an association, correct time order and coherent findings, relative to what is known about the factor and outcome. We demonstrate how these requirements impact epidemiologic investigations into shrimp mortality. Quantifying mortality at intervals throughout growout has been the major problem with on-farm studies. In studies using mortality estimates at the end of production cycles, the correlation of outcome within ponds and within farms reduced our ability to extrapolate results. Measurement error for risk factors has been a secondary problem. Mortality can be measured accurately if the study’s internal time component is a complete production cycle. Because risk factors frequently do not change during a production cycle, the association between risk factors and mortality can be evaluated, assuming a random effect of production cycle, using random-effects logistic regression. It is likely that the farm will modify the cause and effect relationship and future studies should sample multiple farms to increase external validity.

INTRODUCTION

Epidemiologists study the cause of disease in its natural setting. It is a logical extension that a veterinarian studying herd health for shrimp farming must study shrimp diseases using actual farm observations. Epidemiologists avoid defining the word cause, preferring to use words such as determinants, exposures and risk factors. Alternatively, they categorize causes as direct or indirect; necessary or sufficient; and single or multiple, rather than defining cause. In an effort to be pragmatic, Susser (1991) has defined cause as “something that makes a difference” or alters an outcome.

The philosophical view of what constitutes a cause has not been so pragmatic (Rothman 1986). However, some consensus has emerged from the arguments of philosophers. Epidemiologists concur that Koch’s postulates are much too rigid (Susser 1991). Epidemiologists also agree that causes must be hypothesized and then tested for rejection but are never accepted as proven (Susser 1991), thus an epidemiologist will be well served by an iconoclastic point of view. Epidemiologic methods are therefore not very friendly. A brave soul will hypothesize a cause for shrimp dying, and epidemiologists try to disprove the hypothesis. After an hypothesized causal relationship survives the challenge of repeated testing, as the cigarette smoking/mortality relationship did (Eysenck 1991), the risk factor becomes accepted as a cause of mortality. Further sophistication, however, will challenge the hypothesis as our understanding grows and we get closer to understanding the deterministic model. For example, cigarette smoking is no longer considered a cause of mortality by epidemiologists because more specific carcinogens found in cigarette smoke have been identified. Epidemiologists use three rules to reject an hypothesized cause (Susser 1991). The first is association. If a risk factor does not occur more frequently in diseased animals, then it is rejected as a cause. The second is time order. If the risk factor does not precede the outcome, it is rejected. The third criteria used is common sense or coherence, in all its forms. Risk factors are often rejected based on what is known or believed about the cause and outcome.

In epidemiology, two outcomes are frequently studied: morbidity and mortality. The study of morbidity for shrimp culture can be mediated quite effectively by using growth rate as a surrogate measure of wellness (Thompson et al. 1994b). While slow growing shrimp may feel quite well and never miss a day’s work, most producers will accept growth rate as a measure of morbidity because of its correlation to their economic health. However, the study of the causes of shrimp mortality has been fraught with difficulties. There is no lack of hypothesized causes but hypothesis testing is difficult. The objective of this review is to present and discuss examples illustrating the difficulties in hypothesis testing for shrimp mortality from on-farm observations. We present and discuss one example for each of the three criteria for causation: association, time order, and coherence of results.

EXAMPLE 1: ASSOCIATION

INTRODUCTION

Necrotizing hepatopancreatitis (NHP) has been an im-
of shrimp culture in Texas since it was first recognized in 1985 (Frelier et al. 1992). The disease has resulted in mass mortalities of *Penaeus vannamei* in commercial growout ponds. The etiologic agent has been shown to be a pleomorphic Rickettsia-like intracellular bacterium (Frelier et al. 1992). Previous studies have shown the disease to occur at high salinity (20 to 40 ppt), but the role of salinity remains unclear (Frelier et al. 1993). The objective of this study was to describe the association among pond differences in salinity and mortality in 1993 on a Texas farm for which the disease agent was endemic.

**MATERIALS AND METHODS**

A production unit in south Texas that had suffered recurrent problems of NHP was surveyed for histological lesions of NHP during the 1993 production season. Salinity was recorded in each pond on a weekly basis. The mean was calculated for each pond and classified as high salinity if greater than 35 ppt and low salinity if less than 35 ppt. Mortality was calculated as a proportion, with the numerator the difference between the number of shrimp stocked into the pond minus the number harvested from the pond. The denominator was the number of shrimp stocked into the pond. Analysis of the association between salinity and mortality while accounting for clustering of mortality within the pond was performed using various methods described by Donner (1993).

**RESULTS**

**DISCUSSION**

The choice of an appropriate statistical method to test for association was considered. The outcome, mortality, is highly clustered within ponds creating extrabinomial variation. Similar effects are well-described on the smaller scale and are often referred to as litter effects (Donner 1993). The use of the Pearson chi-square statistic ignores the clustering within ponds; this statistic requires that the outcomes be independent and its value overinflates the statistical significance. The t-test methods will evaluate a single measurement of survival from each pond. This will account for clustering but will almost surely violate the assumptions that the variances from each group are normal and homoscedastic. The non-parametric approach is valid but is inefficient because it ignores the number of shrimp in each pond and the magnitude of the differences. Also, the size of the effect is not intuitively useful.

Use of random effects models has intuitive appeal (Curtis et al. 1993). The effects of “pond” can be modeled as a random effect, represented by a distribution of pond effects. By so doing, the variance and standard errors of the estimates are inflated to account for the extrabinomial variation. Software *Egret*® has been marketed to model such random effects. However, the models were not developed with shrimp ponds in mind and most of the fitting algorithms failed with our example. We achieved convergence using the beta-binomial random effects model in *Egret*. By repeating the fitting with different initial values, we observed multiple maxima (several possible solutions). It was straightforward to pick the best of the observed solutions by selecting the model with the lowest deviance (Hosmer and Lemeshow 1989). However, the possibility of an alternative, superior solution, exists. We found no adequate single test for association with the described example.

The evidence of an association between salinity and mortality was strong even though the difference between high and low salinity definitions was very small. Evidence for the association is convincing, but how strong is the evidence that salinity is a cause? Time order has not been established. It is possible that mortality precedes high salinity. High mortality may have been a specific condition associated with a condition that preceded an altered rate of exchange of water. It is unlikely that time order can be established until accurate measures of mortality during a production cycle are possible. The size of the effect makes the likelihood of the association being explained by a bias less likely than if the size of the effect was small.

Proposing salinity as a cause of mortality in the presence of NHP lesions lacks sophistication. What about salinity as a cause of death (Lester and Pante 1992)? Is it its effect on osmoregulation? Is it a stress that reduces immunity? Is it a marker for a change in pond biota with a proliferation of a specific intermediate host? The evidence for coherence in all its forms can be improved with further study. With existing limitations, it is unlikely that we can strengthen the time-order relationship with pond observations. Tank observations may not be applicable to studies on disease in production systems. It is conceivable that increasing salinity in a laboratory tank will not constitute stress but in a pond, high salinity with a combination of other factors will. Tank studies may remove intermediate hosts from the other pond biota. The answer to elucidating the causal mechanism lies in the posing and investigating of more specific and sophisticated epidemiologic hypotheses that will add to our knowledge of the other contributing factors. In the meantime, in a pragmatic world, we must consider salinity as a cause of NHP (Susser 1991).

**EXAMPLE 2: TIME ORDER**

*Vibrio* spp. are considered the most important bacterial pathogens in shrimp culture (Vera et al. 1992), and are important causes of enteritis in humans. Special concern must be placed on pathogens of human concern that may appear in harvested products. Like bacterial pathogens in
other farmed species (Martin et al. 1987), these bacteria are ubiquitous and disease is triggered by stress (Lightner 1993). Stress is frequently implicated by epidemiologists as an initiating factor for bacterial disease in livestock.
in production ponds (Thompson et al. 1994a) and it may serve as a suitable surrogate measure of stress. Concentrations of *Vibrio* spp. vary widely among ponds, and also temporally within ponds. Because of the variance that occurs within pond-cycles, analysis of the *Vibrio/dissolved oxygen* interaction will require a selection of an internal time component and periodic measurement of mortality during a production cycle. The objective of this study was to determine if the presence of *Vibrio* spp. in the hepatopancreas was associated with mortality and if this association was modified by low dissolved oxygen concentrations.

**MATERIALS AND METHODS**

Dissolved oxygen concentrations were performed as close to 6 am. as possible each day. The mean was calculated for the seven measurements of the wk. For categorical analyses, low dissolved oxygen concentration was defined as less than 5.2 mg/L (the median) and high concentration as a mean greater than or equal to 5.2 mg/L. To quantify the number of *Vibrio* colonies, 9 ml of 0.9% saline were mixed with 1 g of hepatopancreas. The mixture was macerated, mixed and a 0.1 ml aliquot was plated on TCBS agar. At 24 h, the number of colonies was counted and classified as high if greater than the median observation (500 colonies) and low if equal to or less than 500 colonies. To determine the pond’s population, a cast net that sampled approximately 1 m² was cast at 54 points. The average number caught was multiplied by 10,000 to give the number per ha and then multiplied by the number of ha. Pond variables and pond population estimates were stored in a Lotus data base and retrieved at the end of the study. To calculate the risk function, the force of mortality was calculated (Kleinbaum et al. 1982). For logistic analyses, the force of mortality was classified as high if positive and low if zero or negative. Multiple logistic regression was performed regressing the outcome high vs. low mortality against pond; pond density and the square of the pond density; phase of the moon; dissolved oxygen; hepatopancreas *Vibrio* concentration and the dissolved oxygen and *Vibrio* interaction. The phase of the moon was modeled as a risk factor if the full moon occurred during the observation wk. Dissolved oxygen and number of *Vibrio* colonies were modeled using the classification variables.

**RESULTS**

Weeks with high mortality were associated with pond, weight, density and the density squared, and occurrence of a full moon during the wk (Table 2). High mortality was also related to the classification of oxygen concentration but not to the number of *Vibrio* colony forming units (CFUs) nor the interaction between dissolved oxygen and *Vibrio* CFUs. A univariate table for the risk of low dissolved oxygen was created (Table 3), which demonstrated that the crude odds ratio for low dissolved oxygen was 1.27. This table was corrected for a non-differential misclassification bias of sensitivity of 0.6 and specificity of 0.6. The true, population crude odds ratio can be shown to be 15.1 (Table 4).

**DISCUSSION**

We have failed to demonstrate a significant effect modification between *Mbutio* and dissolved oxygen concentration. This failure occurred with methods designed to estimate a population size at the beginning and ending of each week. These measurements were made with error that produced a large misclassification bias (Brenner and Blettner 1993). We are not certain of the degree of misclassification but any rise in population estimates constitutes a misclassification and counting these demonstrates the sensitivity of classification to be approximately equal to 0.6. We also believe that if we were classifying a realistic, low mortality risk (0.05/wk), the classification would also have a very low specificity. If the measurement error is centered on zero and normally distributed, we would expect the same percent of errors for both high and low mortality estimates and the specificity would also be equal to approximately 0.6. We show that using a sensitivity of 0.6 and specificity of 0.6 for the misclassification would have a very large bias of the odds ratios toward the null value, We argue that this misclassification biases all potential predictors of mortality toward the null value.

This study was doomed to failure for the detection of relatively small odds ratio. It can be concluded that the odds ratios determined for shrimp density, lunar phase and low dissolved oxygen concentrations were either extremely large or the product of other biases. Our calculations for corrected odds ratios were based on the assumption that the misclassification of mortality was not related to any of the predictors. This assumption could not be validated (Flegal et al. 1991). It is quite possible that estimates may also be related to shrimp density and weight because of shrimp behavior in lunar phases (Griffith and Wigglesworth 1993). It is also possible that low dissolved oxygen concentrations reduce the likelihood of capturing shrimp. In general, the uncertainty of the size of the odds ratios and the size of the misclassification and whether the classification is non-differential or not makes the study design unworkable (Flegal et al. 1991). We believe that measurement error of population estimates during a production cycle makes the study of small or moderate risks untenable.

**EXAMPLE 3: COHERENCE**

**INTRODUCTION**

Taura syndrome was first recognized as a cause of mortality in June 1992 on shrimp farms near the mouth of the Taura River in Ecuador (Brock et al. 1994). Since then,
the disease has been diagnosed elsewhere in Ecuador, Peru, Columbia, Honduras and Hawaii (Brock et al. 1994). In May and June of 1995, five Texas shrimp farms suffered devastating losses in association with a histopathologic diagnosis of Taura syndrome virus (TSV). Because the virus had been considered exotic to Texas, an epidemiologic investigation was conducted to evaluate the most likely of a number of specific possible sources of the virus using case-control methodology (Fonseca and Armenian 1991). Of special concern was the possibility that the spread of TSV was mediated through transport of infected post-larvae.

MATERIALS AND METHODS

All major shrimp growout production units in Texas (n=6) and an experimental unit were visited and evaluated for exposure to the potential sources of infection. Identification of unexpected mortality was based on a questionnaire delivered by the lead investigator to the farm owners or managers. Each unit was evaluated for the presence of TSV based on histopathology. Cases were farms with unexpected mortality and a histopathologic diagnosis of TSV. Exposure to the potential sources was based on historical data and inspection by the lead investigator. Associations between potential infection sources (factors) and case status were evaluated with Fisher’s Exact Test.

RESULTS

Taura syndrome was diagnosed in five of six commercial growout farms and was absent in the experimental unit. Feed used consisted of six different formulations from multiple batches from two separate feed companies. Water inlets were closed for the majority of the case ponds. It became apparent during the outbreak that new cases occurred irrespective of water flowing into the ponds. The index case farm also supplied larvae to other farms in Texas (and elsewhere). Three case farms received larvae when TSV was active on the index farm (after May 6). This source of larvae did not explain disease at the index farm and at one other case-positive farm. Exposure to airborne factors, wild in the Laguna Madre, was found at all farms but not the experimental unit.

DISCUSSION

Identification of a causal factor requires the demonstration of association, temporal sequence and coherence. However, we must consider that our general grouping of causes into airborne, waterborne, feedborne and animalborne is likely an exhaustive list and to meet our objective we should identify the most likely source. If we hypothetically expand our sample to include 10 more US shrimp farms, all newly sampled farms would be factor negative and case negative. If this sampling did not constitute a bias, we would conclude that the association of transported post-larvae and TSV were associated; however, the same can be said to be true of exposure to the Laguna Madre and TSV. For temporal sequence, we can say both factors preceded the mortality effect.

In our examination of coherence, we note inconsistencies in the lag (or incubation) periods. Incubation periods have been long recognized as useful indications of a cause (Sartwell 1993). For the three farms receiving larvae as potentially infected, one had a 1 wk incubation period and two had 5 wk incubation periods. The incubation periods for the airborne source can, in contrast, be explained by a south to north migration of the vehicle. Analogy, a form of coherence, also favors the airborne source hypothesis. Within case farms, spread was rapid among ponds with a very short incubation and a short period of mortality. The findings are compatible with a common source airborne spread. This could be explained by multiplication of a virus within an insect host but not by a mechanical vector. If the virus was spread by infected larvae, the hypothesis of airborne-spread must still be true within farms. Because of the rapidity of spread, we propose that the infection source is part of the preferential diet of shrimp such as an insect with both airborne and waterborne life stages. Through analogy and the knowledge that within-farm spread was airborne, it was shown that airborne-spread was definitely involved. Was the spread by infected post-larvae also involved? The preponderance of other evidence (coherence) says no.

CONCLUSIONS

The studies of shrimp diseases with on-farm observations have been frustrating. We propose the following recommendations for future study.

Concentrate on “fixed” factors as risk factors. Risk factors that are constant throughout a growout cycle by definition must precede mortality so time order cannot be faulty. These factors include: salinity and other environmental variables on most farms; pond size, depth and other physical properties, including aerators; and endemic diseases.

Use random effects models to demonstrate associations. These models can account for extrabinomial variation. Although developed for clusters much smaller than shrimp ponds, these models can be used provided routine diagnostics for regression models are applied.

Increase the number of farms in the sample. Fixed risk factors will usually be constant for all ponds within a farm. Random effects models are used when the outcomes are clustered. A farm becomes a single cluster with a single outcome rate and a single level of fixed risk factor. The need for multiple farms is obvious.

LITERATURE CITED


Diarrhetic Shellfish Toxins Determined by High-Performance Liquid Chromatography-Fluorometry in Mussels, *Mytilus coruscus*, from the Niigata Coast of Japan

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**ABSTRACT**

Determination of okadaic acid (OA) and its analogues, *dinophysistoxin*-1 (DTX-1) and *dinophysistoxin*-3 (DTX-3), in mussels collected from the Niigata coast of Japan was carried out by high-performance liquid chromatography (HPLC) with fluorometric detection after derivatization with 9-anthryldiazomethane. Analyses of toxic material indicated the presence of OA and DTX-1; however, the toxicity determined by HPLC-fluorometry was far below that estimated from mouse bioassays, suggesting the presence of other toxins.

**INTRODUCTION**

Diarrhetic shellfish poisoning (DSP) has been recognized as a worldwide problem since it was reported by Yasumoto *et al.* (1978). Among the diarrhetic shellfish (DS) toxins derived from dinoflagellates, *Dinophysis* spp. and *Prorocentrum* spp. (Lee *et al.*, 1989), the most important toxins responsible for diarrheal symptoms are okadaic acid (OA) and its derivatives, *dinophysistoxin*-1 (DTX-1) and *dinophysistoxin*-3 (DTX-3) (Lee *et al.*, 1987). In 1994, a DSP outbreak occurred along the Niigata coastal area, Japan. Shellfish harvesting was prohibited from May 16 to August 2. The present paper reports results of DS-toxin analysis by high-performance liquid chromatography (HPLC)-fluorometry to elucidate the nature of the toxic DSP compounds in mussels collected from the Niigata coastal area. Determination by HPLC-fluorometry of the free fatty acids (FFAs) which have been suggested to interfere with mouse bioassays (Takagi *et al.* 1984) is also described.

**MATERIALS AND METHODS**

Mussels, *Mytilus coruscus*, were collected from the Niigata coastal area during the period May 30 to June 27, 1994. Five individual mussels were used for each analysis. Acetone extraction of DSP toxins from the midgut glands of mussels and the following mouse bioassays were carried out using the procedure described by Yasumoto (1981). Analyses of OA and DTX-1 in the acetone extracts by HPLC-fluorometry were carried out according to the method of Lee *et al.* (1987). Determination of DTX3 was carried out by the detection of DTX-1 obtained from DTX-3 via alkaline hydrolysis (Suzuki 1994) after separation by partitioning between 80% methanol (OA and DTX-1) and n-hexane (DTX-3). Fluorescent peaks corresponding to the 9-anthryldiazomethane (ADAM) derivatives of OA and DTX-1 were confirmed by a second HPLC-fluorometry using a Capcell Pak CN SG120 column (4.6 mm ID x 250 mm; Shiseido, Tokyo, Japan) as described by Zhao *et al.* (1993). Further confirmation of the OA and DTX-1 peaks was carried out by HPLC-fluorometry using a LiChrosorb RP-18 column (4.0 mm ID x 250 mm; Merck, Darmstadt, Germany), with acetonitrile-methanol-water (8: 1: 1, v/v/v) as the mobile phase and a flow rate of 1.1 ml/min at 35°C. Determination of the peak area corresponding to OA and DTX-1 was carried out on the second HPLC run equipped with a Capcell Pak CN SG120 column. Determination of FFAs by HPLC-fluorometry was carried out according to the method of Suzuki (1994). The toxicity of the FFAs was calculated from data reported by Takagi *et al.* (1984). One liter seawater samples were collected at 8 m depth at the monitoring station and fixed with formalin (5%). The solution was concentrated to 10 ml for counting and identification of *Dinophysis* spp. by microscopy.

**RESULTS AND DISCUSSION**

Table 1 shows the mouse bioassay results for the midgut glands of mussels during the DSP outbreak and cell densities (cells/L) of *Dinophysis* spp. from 8 m depth where the mussels were harvested. The DSP outbreak on May 9,
Table 1. Results of mouse bioassays and cell densities (cells/L) of *Dinophysis* species collected from the Niigata coastal area in 1994

<table>
<thead>
<tr>
<th>Date</th>
<th>Bioassay Results$^1$ (MU $^2$/g)</th>
<th>D. fortii</th>
<th>D. mitra</th>
<th>D. rudgei</th>
<th>Other spp.$^3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>May 9</td>
<td>0.5 - 1.0</td>
<td>170</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>May 16</td>
<td>0.5 - 1.0</td>
<td>10</td>
<td>—</td>
<td>10</td>
<td>—</td>
</tr>
<tr>
<td>May 22</td>
<td>0.3 - 0.5</td>
<td>10</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>May 30</td>
<td>0.5 - 1.0</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>June 6</td>
<td>0.5 - 1.0</td>
<td>10</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>June 14</td>
<td>0.5 - 1.0</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>June 20</td>
<td>0.5 - 1.0</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>June 27</td>
<td>0.5 - 1.0</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>July 8</td>
<td>&lt;0.3</td>
<td>—</td>
<td>30</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>July 19</td>
<td>&lt;0.3</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>July 26</td>
<td>&lt;0.3</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

$^1$Toxicity of the mussel midgut glands.

$^2$Mouse unit (MU); one MU is defined as the amount of toxins to kill a male mouse of ddY strain of 20 g body weight in 24 h.


1994, occurred with the appearance of *D. fortii*, suggesting that *D. fortii* was responsible for the observed toxicity of mussels along the Niigata coast in 1994. The toxicity of mussels remained above the quarantine level of 0.5 mouse unit (MU)/g of midgut glands from May 9 to June 27.

Fig. 1 shows a representative HPLC profile of the ADAM derivatives of extracts obtained from the midgut glands of mussels. Peaks corresponding to OA and DTX-1 were detected, whereas the fluorescence intensity of respective peaks was very small (Fig. 1). Peaks corresponding to OA and DTX-1 were collected from the outlet of the fluoromonitor, and each concentrated fraction was verified by the two different second chromatography runs, using a Capcell Pak CN SG120 (Zhao et al. 1993) or LiChrosorb RP-18 column. Chromatograms of the fractions of OA and DTX-1 collected from the first HPLC indicated the presence of peaks with retention times exactly matching those obtained from the ADAM derivatives of the standard toxins (Figs. 2, 3), indicating that the mussels under investigation were contaminated by OA and DTX-1. In samples of toxic Japanese mussels, the most prominent DS toxin is usually DTX-1 (Kumagai et al. 1986). The presence of OA in Japanese mussels was confirmed by HPLC-fluorometry. DTX-1 hydrolyzed from DTX-3 was not detected in the present HPLC-fluorometry, indicating that mussels analyzed did not contain DTX-3.

Table 2 gives the concentrations and toxicities of OA and DTX-1 in the midgut glands of mussels as determined by HPLC-fluorometry. The sum of the toxicity of OA and DTX-1 was 0.02-0.13 MU/g of midgut glands. The tox-
Fig. 2. HPLC profiles of the ADAM derivatives on the Capcell Pak CN SG120 column. (A) purified OA and DTX-I; (B) fraction corresponding to DTX-I in HPLC on the Develosil ODS-5 column; (C) fraction corresponding to OA in HPLC on the Develosil ODS-5 column. Conditions: mobile phase, acetonitrile-water (53:47, v/v); monitor, excitation 365 nm, emission 412 nm; flow rate, 1.1 ml/min; temperature, 35°C.

Fig. 3. HPLC profiles of the ADAM derivatives on the LiChrosorb RP-18 column. (A) purified OA and DTX-I; (B) fraction corresponding to DTX-I in HPLC on the Develosil ODS-5 column; (C) fraction corresponding to OA in HPLC on the Develosil ODS-5 column. Conditions: mobile phase, acetonitrile-methanol-water (8:1:1, v/v); monitor, excitation 365 nm, emission 412 nm; flow rate, 1.1 ml/min; temperature, 35°C.

The FFA content is also shown in Table 2. Although a marked increase in FFA content was observed on June 20 and 27, the FFA content was not sufficient to give positive results (>0.5 MU/g) in the mouse bioassays.

The presence of OA and DTX-I in toxic mussels collected from the Niigata coast was confirmed by HPLC-fluorometry, but the sum of the toxicities determined by HPLC-fluorometry was too low to account for the mouse lethality. FFA contents were also insufficient to interfere with the mouse bioassay. These results indicate the presence of other DSP toxins during routine instrument methods of monitoring for the toxicity of mussels in this area.

**LITERATURE CITED**


Analytical Methods for Diarrhetic Shellfish Poisoning (DSP) Toxins and a Study of Toxin Production by *Prorocentrum lima* in Culture

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**ABSTRACT**

Incidents of diarrhetic shellfish poisoning (DSP) pose a serious threat to the aquaculture industry. DSP results from consumption of shellfish contaminated with toxic dinoflagellates such as species of *Dinophysis* and *Prorocentrum*. During a study to determine the kinetics of production and excretion of DSP toxins in culture by one such organism, *Prorocentrum lima*, a number of new ester derivatives of okadaic acid and DTX1 were discovered. It was also found that enzymatically-catalyzed hydrolysis and methanolysis reactions were occurring during simple extractions with aqueous methanol. Extraction and analysis methods developed to determine toxin concentrations in cells and in the medium will be valuable for future studies on the uptake and depuration of DSP toxins by shellfish, and for protection of public health.

**INTRODUCTION**

Diarrhetic shellfish poisoning (DSP) is a severe gastrointestinal illness resulting from consumption of shellfish contaminated with toxigenic dinoflagellates, such as certain *Dinophysis* and *Prorocentrum* species (Yasumoto and Murata 1990). The main toxins responsible for DSP are okadaic acid (OA) and the dinophysistoxins, DTX1 and DTX2 (1-3, see Fig. 1). These compounds have been shown to be potent phosphatase inhibitors, a property which can cause inflammation of the intestinal tract and diarrhea. In addition, OA and DTX1 have been shown to have tumor-promoting activity. A number of naturally-occurring derivatives of these toxins has also been identified. The 7-O-acylated (C to C) derivatives (4) of 1-3 have been found in shellfish tissue and designated as DTX3 (Yasumoto *et al.* 1989, Marr *et al.* 1992b). However, DTX3 toxins have not been detected in microalgae, suggesting that they are products of shellfish metabolism (Yasumoto *et al.* 1989). Several ester derivatives of OA, such as diol-ester 5, have been isolated from *P. lima* and *P. maculosum* (Yasumoto *et al.* 1989, Hu *et al.* 1992). Although the ester derivatives do not appear to be phosphatase inhibitors, they have the potential to be hydrolyzed readily in the digestive tract to yield an active parent DSP toxin. Recently, a water-soluble DSP toxin (DTX4) was isolated from an eastern Canadian strain of *P. lima* (Hu *et al.* 1995). This compound is a complicated derivative of OA, in which the primary hydroxyl of diol-ester 5 is esterified with a trisulfated end group.

In this study we report on the DSP toxins produced by the dinoflagellate *P. lima* in culture. A number of new ester derivatives of OA and DTX1 were identified, and it was found that enzymatically-catalyzed transformations were occurring during simple extractions with aqueous methanol. New analytical procedures are demonstrated for the accurate determination of toxin concentrations in cells and in the medium.
MATERIALS AND METHODS

CULTURING PROCEDURES

The strain of _P. lima_ used in this study, its isolation, culture medium and growth conditions were reported previously (Marr et al. 1992a, Jackson et al. 1993, McLachlan et al. 1995).

SAMPLE PREPARATION

Aliquots (50 ml) of _P. lima_ culture were transferred to 50-ml plastic centrifuge tubes and centrifuged for 10 min at 6600 × g. The supernatant was decanted without disturbing the cell pellets. Several different extraction methods were investigated, the principal ones being:

Method 1 (80% methanol): Each cell pellet was resuspended in 2 ml of methanol/water (8:2) and sonicated for 1 min in pulse mode (50% duty cycle, 375 W) while cooling in an ice bath. After centrifugation for 10 min at 6600 × g, the supernatant was decanted. The pellet was rinsed twice (vortex mixing, centrifugation) with 1 ml methanol/water (8:2). Supernatants were combined and adjusted to 5.0 ml. Extracts were passed through a 0.45-μm filter prior to analysis.

Method 2 (French press): Four cell pellets were each resuspended in 0.2 ml 50 mM TrisHCl pH 7.4, combined and passed through a chilled French press at pressures >10 Kpsi. A 1 ml aliquot of buffer was used to wash remaining residues through the press and the sample was then brought to 2.0 ml with buffer. Aliquots (0.5 ml) were mixed with 2.0 ml methanol and processed as in Method 1.

Method 3 (freeze/thaw): Each cell pellet was resuspended in 0.5 ml of TrisHCl buffer and immersed in liquid nitrogen. The sample was allowed to thaw at room temperature and left in the dark for 24 h. Then 2 ml of methanol were added and the sample was sonicated and extracted as in Method 1.

Method 4 (boiling): Each cell pellet was resuspended in 0.5 ml of TrisHCl buffer and immersed in boiling water for 3 min. Following this, 2 ml of methanol were added followed by sonication and extraction as in Method 1.

CHEMICAL ANALYSES

Analyses of 1-3 and 5 were performed by positive ion-spray liquid chromatography-mass spectrometry (LC-MS) and of 1-3 by LC with fluorescence detection (FLD) after derivatization with anthryl diazomethane (ADAM), as previously reported (Quilliam 1995). The DTX4 and related toxins were analyzed using the negative ion mode, a 2 x 150 mm column packed with 5 mm Zorbax Rx-C8, 0.2 ml/min flow rate and gradient elution with an aqueous acetonitrile-ammonium acetate (1 mM, pH 7) mobile phase programmed from 20% to 50% acetonitrile over 15 min. Compounds 5 and 6 were also analyzed by LC with UV detection at 238 nm using the same column and mobile phase.

RESULTS AND DISCUSSION

Published extraction methods for DSP toxins in plankton samples have used solvents such as methanol, acetone and chloroform. Initially, we sonicated cells isolated by centrifugation in aqueous 80% methanol and followed this by LC-FLD analysis of the ADAM derivatives. This gave excellent reproducibility with replicate subsamples of a single culture in early growth phase. However, erratic results in the cellular concentration of OA (but not DTX1) were observed as the methods were applied in a study of the rate of toxin production in batch culture. Results of one such experiment are shown in Fig. 2. It was evident from literature reports that others had also experienced erratic quantitative results in such experiments (Jackson et al. 1993, McLachlan et al. 1995), although it was assumed that these arose from difficulties with the LC method used. However, as reported below, we have now determined that such results are artifacts of sample preparation and enzyme action.

The positive ion-spray LC-MS analysis of a 70-day-old culture sample (Fig. 3a) extracted using Method 1 showed the presence of OA (peak 1), DTX1 (peak 2) and OA diol-ester (peak 5). The diol-ester of DTX1 was not
observed at significant levels in this particular isolate, although we have observed it in other *P. lima* isolates (unpublished results). At first, it was considered that the n-reproducible results were due to cells not being adequately ruptured by sonication, but repeated extraction of sonicated cells resulted in no further extraction of toxins.

Cells could be rapidly and completely disrupted by passing them through a French press, and subsequent methanolic extraction showed increased levels of OA, DTX1 and OA diol-ester (Fig. 3b). If the disrupted cells were held at room temperature for several h, however, the diol-ester concentration decreased while that of OA increased. Similar results were observed if a cell pellet was resuspended in Tris-HCl buffer, frozen in liquid nitrogen and allowed to thaw. Fig. 3c shows the results after freezing and then incubating at room temperature for 24 h. All of the diol-ester had disappeared, leaving only OA and DTX1. These observations suggested enzymatic hydrolysis reactions.

A peak at 5.3 min in the m/z 819.5 mass chromatogram (peak 1 m, Fig. 3a) was identified as being due to the methyl ester of OA. The level of this compound was highly variable even between replicate subsamples of culture, and was sometimes greater than that of OA. The amount of methanol used in the extraction step had a marked effect, with lower percentages increasing the level of methyl ester, opposite to what would be expected if there was a problem with extraction yields due to lipophilicity. This suggested a reaction between methanol and the analytes. Different amounts of water in cell pellets during extractions could partially explain the variations in methyl ester levels observed previously. Substitution of deuterated methanol (CD$_3$OH) in the extraction protocol showed a shift of the m/z 819.5 ion to m/z 822.5, proving that the methyl ester was an artifact of the extraction procedure. This conclusion was substantiated by substitution of acetonitrile or tetrahydrofuran for methanol, with elimination of methyl ester formation. However, yields of OA and its diol-ester proved much lower with these solvents for reasons which became apparent after the discovery of DTX4 (see below). When methanol was added to the French press homogenates and the mixtures allowed to incubate, methyl ester could be detected. At 40% methanol, nearly complete conversion of compound 5 to OA methyl ester occurred. This observation, along with the lack of conversion in boiled extracts (Fig. 3d), led to the conclusion that hydrolysis and methanolysis of 5 in these extracts were occurring enzymatically. Immersing the cell pellet in boiling water for 3 min prior to extraction with methanol eliminated the methyl ester formation, but under these conditions the diol-ester concentration was very low (Fig. 3d).

At this point, information on the existence of DTX4 became available (Hu et al. 1995). An additional peak close to the solvent front in the m/z 805.5 chromatogram (peak 6f, Fig. 3) could then be explained. It is now known that this signal is due to a weak positive fragment ion from DTX4. The peak is most intense in the boiled cell extract. A specific LC-MS method for the analysis of DTX4 has now been developed. Fig. 4 shows the negative ion full-scan LC-MS analysis of the same extract of boiled cells analyzed in Fig. 3d. DTX4 was easily detected by [M-3H]$^-$ and [M-2H]$^-$ ions. Interestingly, a number of related new compounds were also detected. The signals from these compounds are evident in the other reconstructed mass chromatograms in Fig. 4 and peak identities are provided in Table 1. Most of the structural variations appear to be associated with the sulfated end group as only one diol-ester of OA is observed in this *P.lima* isolate. Some derivatives of DTX1 are also observed but at much lower concentrations than those of OA, OA and DTX1 can also...
Table 1. DSP toxins observed in *Prorocentrum lima* extracts.

| Gpd. # | Mol. wt. | Ions observed (m/z)* | Toxin assignment** | Structure of compound
|--------|----------|----------------------|-------------------|------------------------
| 1      | 804.5    | 805.5 (+); 803.5 (-) | Okadaic acid (OA) | not available
| 2      | 818.5    | 819.5 (+); 817.5 (-) |                | not available
| 5      | 928.6    | 929.6; 805.5 (+)     |                | not available
| 6      | 1472.6   | 489.9; 735.3; 803.5 (-) |                | not available
| 7      | 1486.4   | 494.5; 742.3; 817.5 (-) |                | not available
| 8      | 1488.6   | 495.2; 743.3; 803.5 (-) |                | not available
| 9      | 1504.6   | 500.5; 751.3; 803.5 (-) |                | not available
| 10     | 1514.6   | 503.9; 756.3; 803.5 (-) |                | not available
| 11     | 1518.6   | 505.2; 758.3; 817.5 (-) |                | not available
| 12     | 1552.6   | 387.2; 516.5; 775.3; 803.5 (-) |                | not available
| 13     | 1568.6   | 391.2; 521.9; 783.3; 803.5 (-) |                | not available
| 14     | 1576.6   | 524.5; 787.3; 803.5 (-) |                | not available
| 15     | 1584.6   | 395.2; 527.2; 791.3; 803.5 (-) |                | not available
| 16     | 1598.6   | 398.7; 531.9; 798.3; 817.5 (-) |                | not available
| 7c     | 1486.4   | 494.5; 742.3; 817.5 (-) |                | not available
| 8c     | 1488.6   | 495.2; 743.3; 803.5 (-) |                | not available
| 9c     | 1504.6   | 500.5; 751.3; 803.5 (-) |                | not available
| 10c    | 1514.6   | 503.9; 756.3; 803.5 (-) |                | not available
| 11c    | 1518.6   | 505.2; 758.3; 817.5 (-) |                | not available
| 12c    | 1552.6   | 387.2; 516.5; 775.3; 803.5 (-) |                | not available
| 13c    | 1568.6   | 391.2; 521.9; 783.3; 803.5 (-) |                | not available
| 14c    | 1576.6   | 524.5; 787.3; 803.5 (-) |                | not available
| 15c    | 1584.6   | 395.2; 527.2; 791.3; 803.5 (-) |                | not available
| 16c    | 1598.6   | 398.7; 531.9; 798.3; 817.5 (-) |                | not available

*a*Positive or negative ionization indicated by (+) or (-) following ions.

*Identification of compounds 1, 2, 5 and 6 is given in Figure 1.

*Compounds 7, 11 and 16 are derivatives of DTX1 (methyl group at C35).

be determined in the same analysis, although at lower sensitivity than for DTX4. An LC-UV method for detecting DTX4 and the diol-ester was also developed which allowed for the rapid quantitation of these compounds in algal extracts.

Analyses revealed that DTX4 was the most abundant of the DSP toxins in boiled *P. lima* cells, yet was present at very low concentrations in fresh French press extracts. A corresponding increase in diol-ester concentrations in the French press homogenates (Fig. 3b) suggested that enzymatic conversion of DTX4 to diol-ester was occurring within min. Freeze/thaw treated samples also had low DTX4 levels in contrast to those in boiled extracts, providing further evidence for enzymatic hydrolysis of DTX4. It was observed that the cells store most of the DSP toxins in the DTX4 form which is first enzymatically hydrolyzed to the diol-ester when cells are disrupted and then mom slowly hydrolyzed to OA. From these observations, it appears that DTX4 and the enzyme(s) responsible for its hydrolysis are sequestered in different compartments in the cell.

The ability to analyze selectively for the suite of DSP toxins and restrict enzymatic hydrolysis using the boiling technique was applied to study the kinetics of DSP toxin production and excretion by *P. lima* in culture. Analysis of independent batch cultures over a 90-day period revealed clear and consistent trends in the intracellular levels of all the toxins (Fig. 5). Total intracellular toxin levels in culture increased linearly with time throughout the culture period. On a per cell basis, a positive correlation with growth rate is indicated. DSP toxin production is therefore not disassociated from growth, as is the case with many
secondary metabolites. After cessation of cell growth, toxin production continued, resulting in progressively higher intracellular levels, reaching 40 fmole/cell after 90 days. DTX4 is clearly the dominant intracellular DSP component, exceeding the levels of all other toxins combined. Both OA and DTX1 were present in about equal amounts; however, only trace levels of OA diol-ester were present. OA and, to a much lesser extent, DTX1 were found to accumulate in the medium while only trace levels of DTX4 and the diol-ester were detected (Fig. 6). Release of OA into the medium is probably enhanced by the high aqueous solubility of DTX4 and mediated by the enzymatic pathway described above. This could explain why OA is present at much lower concentrations in the medium than OA even though the intracellular concentrations were similar. Evidence for the role of the hydrolytic enzymes in OA excretion is suggested by the lack of other DSP compounds in the medium and by the rapid conversion of DTX4 to OA-diol-ester upon cell disruption, followed by enzymatic conversion of the diol-ester to OA. Since OA is a potent inhibitor of eukaryotic phosphatases, the excretion of DTX-4, and thus ultimately OA, by P. lima may constitute a chemical defense system directed at predators, competitors, or pathogens (Windust et al. in press).

CONCLUSIONS

We are currently investigating the fate of the diol-ester and DTX4 derivatives in shellfish. We hypothesize that they are hydrolyzed to OA and DTX1 due to esterases in the shellfish digestive gland as well as those from the algae. Although the diol-ester is not toxic, hydrolysis yields OA. Therefore, comprehensive analysis of all toxin-related compounds is necessary to properly assess the toxicity of any new plankton isolate. Clearly LC-MS will be an important tool for this task. The fingerprinting of the suite of toxins is also of interest for chemotaxonomic studies. In preliminary experiments, we have seen tremendous variations in toxin profiles among different isolates of P. lima and between different species of Proorocentrum. For those laboratories without access to LC-MS equipment, the freeze/thaw/hydrolyze method should be useful for assessing the toxic potential of plankton samples and culture material. Experiments directed toward the accumulation and depuration of toxins in shellfish using P. lima should be carefully designed to avoid the analytical errors that are possible due to enzymatic transformations.

ACKNOWLEDGMENTS

We thank W.R. Hardstaff and A. Reeves for technical assistance and J.L.C. Wright for a sample of DTX4. This research was supported in part by DFO/NSERC subvention and NSERC operating grants awarded to JLM.NRCC No. 39692.
LITERATURE CITED


Osteological Evaluation in Artificial Seedlings of *Paralichthys olivaceus* (Temminck and Schlegel)

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**ABSTRACT**

As one of the approaches to clarify the morphological characteristics in artificial seedlings, the axial skeleton of the Japanese flounder, *Paralichthys olivaceus* (Temminck and Schlegel), was observed. Basically, the axial skeleton of *P. olivaceus* is composed of 38 total vertebrae, viz, 11 abdominal and 27 caudal vertebrae. However, in artificial seedlings, the number of total vertebrae was fewer than that in wild fish, and the constitution of vertebrae was also more variable (mean ± SD = 36.4 ± 2.5). The frequency of central fusion was 63% in abdominal and 74% in caudal vertebrae in artificial seedlings, while it was 0% in abdominal and 4% in caudal vertebrae in wild fish. In addition, each symptom of fusion in artificial seedlings was generally serious. Concerning the frequency of central fusion in artificial seedlings, there was approximately one gentle peak in abdominal vertebrae and three peaks in caudal. The completion of central development was the fastest in the basioccipital articulatory process, the second in the urostyle, while the last in the 25th or 26th vertebrae where a relationship between the frequency of fusion and the velocity of ossification was suggested.

**INTRODUCTION**

In the last two decades, the technique of seedling production has been remarkably improved in Japan. In some important commercial fish species, the mass production of artificial seedlings has become possible. However, in artificial seedlings, morphological malformation constantly appears at high frequency and the quality of seedlings is not at the level of wild fish. The incompleteness of feeding and swimming organs in early development is said to affect the survival rate greatly after the juvenile period, which is an emergent problem to be solved in the production of healthy artificial seedlings. This study examines the developmental changes in the trunk and clarifies the morphological characteristics of artificial seedlings.

The Japanese flounder, *Paralichthys olivaceus* (Temminck and Schlegel), is one of the typical metamorphosing fishes in Japan. In order to elucidate the relationship between osteological development and bone anomaly, some standard structures in the skeletal system of *P. olivaceus* were reported (Hosoya and Kawamura 1993). In this paper, using an axial skeleton which supports the body, the osteological characteristic of artificial seedlings is clarified under comparison with wild fish. That is, the standard structure of an axial skeleton and a bone anomaly typically seen in artificial seedlings are described. Then, from the point of view in osteological development, the factors causing bone anomaly are assumed. Finally, including the information about morphometry, the developmental stages in *P. olivaceus* are rearranged and the relationship between character expression and morphogenesis is discussed as well.

**MATERIALS AND METHODS**

Artificial seedlings of *P. olivaceus* were reared and fed with a series of rotifer, *Terebratalia* and pellets from February to June in 1994 and 1995. Rotifers had beforehand been enriched with a phytoplankton, *Nannochloris oculata*. Rearing experiments were done in a 500 L fish tank at unadjusted water temperature of 15 to 19°C. Seedlings were first reared in stationary water for a week and then transferred to running water (50 L h\(^{-1}\)). Sampling was done twice a week. Collected samples were immediately fixed with 3% formalin, 70% ethanol and *Bouin* solution, respectively. As a control, wild fish were sampled from wild populations in the following areas: the Tone River, Maizuru, Kasumi, Aoya, Tenzin, the Yura River, Kazusa and Tsushima (Fig. 1). The skeletal system was stained with either a double-staining technique (*Kawamura and Hosoya* 1991) or a single-staining technique, using only *alizarin* red S or *alcian* blue, and examined under a binocular microscope. Ossification was histologically confirmed with the appearance of osteocytes. As for anatomical terminology relating to the skeletal system, *Hosoya* (1991) was followed.
and caudal vertebrae were defined as vertebrae equipped with parapophyses or a sharp haemal spine, respectively (Fig. 2). The urostyle was included in the caudal vertebrae.

RESULTS

CHARACTERISTICS OF VERTEBRAL COLUMN IN P. OLIVACEUS

To elucidate the morphological characteristics of artificial seedings with bone anomalies, it is necessary to utilize the accurate information on the skeletal system of healthy wild fish from wild populations. Though P. olivaceus is a commercially important fish species in Japan, the information about its skeletal system is scarce except for the conclusive descriptions about flatfishes by Amaoka (1969) and Sakamoto (1984). The standard structure and the developmental process of the vertebral column in P. olivaceus are described below.

GENERAL MORPHOLOGY

Abdominal vertebrae are expanded up and down. The basic number of abdominal centra supporting the vertebrae is nine. The first abdominal vertebra is articulated with the basioccipital articulatory process (BOAP) at the anterior tip. Each abdominal vertebra is equipped with a neural spine dorsally and a neural arch ventrally, for protecting the notochord (Fig. 2). On the fifth abdominal vertebra or the posterior element, a pair of parapophyses develop ventrally. The first to fourth neural spines are remarkably thick compared with the others, which represents an important characteristic of the vertebral column in P. olivaceus (Fig. 5, upper). Left parapophyses are constantly longer than right ones, probably reflecting an asymmetrical body shape. On the distal end of a parapophysis, a rib is attached. The caudal vertebrae are composed of approximately 27 centra. At the posterior end, they are transformed into the complicated urostyle. Each caudal vertebra is equipped with a pair of long haemal spines ventrally, which form a haemal arch at the base. The first haemal spine is thick and extends ventrally. On its anterior surface, there is a groove receiving the first proximal pterygiophore from the anal fin.

DEVELOPMENTS OF VERTEBRAL COLUMN

The developmental process of the vertebral column in P. olivaceus is described below (Figs. 3, 4) in stages defined by Minami (1982).

Stage A. The skeletal system is underdeveloped. Osteocytes surrounding the notochord are not recognized at all. In the neurocranium, one of the components in the axial skeleton, trabecula cartilages, was stained dark blue with alcian blue.

Stage B. The segmentation of the notochord began in part. As the first bone elements, the first and second neural
Fig. 3. Osteological development of abdominal vertebrae in Paralichthys olivaceus. Each character corresponds to a developmental stage as defined by Minami (1982) except for F, which here divided into early (Fe) and late (Fl) substages.
Fig. 4. Osteological development of caudal vertebrae in Paralichthys olivaceus. *hp*, hypural primordium; *ou*, ossified urostyle; *uoc*, unossified centrum.
arch cartilages appeared. In the neurocranium, cartilages surrounding the otic capsule were apparent. Trabecula cartilages expanded much further.

Stage C. This stage is the first important phase in the development of the vertebral column. Though almost all neural arch cartilages appeared, the arrangement of neural arches was not serial, because the fifth to seventh neural arch cartilages from the posterior end had not yet been formed. Likewise, in the haemal arch series, all cartilage elements appeared. In the dorsal caudal region, two large cartilage masses, hyurnal primordia, were also apparent. Parapophyses and haemal spines were totally underdeveloped. However, the first haemal spine, the longest one located at the anterior end of the vertebral column, was exceptionally stained dark blue with alcian blue. As elements relating to the vertebral column, 10 proximal pterygiophores were recognized at the base of the long dorsal fin rays. With the second to fourth proximal pterygiophores, median pterygiophore cartilages were already articulated.

Stage D. The notochord is straight; however, its posterior end began to bend dorsal. Hyurnal and epural primordia also appeared ventrally just behind the posterior end of the notochord.

Stage E. Tendency of dorsal bending in the notochord has become more apparent. All the parapophyses and the second and haemal spines posterior to the second appeared. All the cartilage elements constituting the vertebral column were present. In the neurocranium, the bottom and frontal regions began to ossify.

Stage F. This stage is the second important phase in development of a vertebral column, viz, ossification drastically proceeds. Concerning cartilage bones, the first to 20th neural spines and the first to 10th haemal spines were stained red with alizarin red S. The degree to be stained was remarkable especially in the first to fourth neural spines and the first to seventh haemal spines. In adult fish, neural arches, neural spines and haemal spines, except the posterior ones in the caudal region, were stained dark red. All the parapophyses were ossified. On the other hand, concerning membrane bones, BOAP was the first to be ossified (Fig. 3), and the urostyle the second (Fig. 4). In addition, each centrum began to be ossified. The ossification degree in the centrum had progressed greatly in the anterior elements of the abdominal vertebrae, and in the seventh to 11th elements of the caudal vertebrae. Contrary to this, the 23rd to 26th caudal vertebrae (the fifth to second preurostyles), in which ossification was strikingly late, formed characteristic unossified centrales (UOC) between the preceding caudal vertebrae and the urostyle (Fig. 4).

Stage G. Each centrum stands together almost in a straight line with a break point identical with the 25th to 26th caudal vertebrae (the third to second preurostyles). Between the neighboring caudal vertebrae, however, a gap of about one third or half width of a centrum was present. The neurocranium began to be transformed. The frontals, the parietals, the sphenotics, the pterotics and the supraoccipitals were recognized, respectively, in it. Each proximal pterygiophore cartilage of the anal fin, articulated with the first haemal spine, was stained dark blue with alcian blue.

Stage H. The skeletal system of the vertebral column was almost completed by the connection of the 25th to 26th caudal vertebrae with the other ossified vertebrae. However, centrales only surrounding the notochord had not yet been constricted. Many pterygiophores in dorsal and anal fins still remained cartilaginous.

COMPARISON OF ARTIFICIAL SEEDLINGS WITH WILD FISH

Meristic counts are known to be strongly affected by environmental factors like temperature during development. From hatching, artificial seedlings are subjected to an environment strikingly different from what they naturally experience. So, it is likely that meristic counts in artificial seedlings are different from those in wild fish. In the following, to determine the number of vertebrae which is a typical meristic count, the frequency of shrinkage and fusion in the centrum of artificial seedlings was compared with wild fish from some localities in Japan.

NUMBER OF VERTEBRAE

In wild fish, the mean of abdominal vertebrae was 11.0, except for a few samples from Maizuru, Kyoto (Table 1). The standard deviation (SD) fluctuated (0.2 in the Yura River, Tottori, 0.3 in Kasumi, Hyogo, 0.6 in Maizuru), while it was stable (0.0 in the other localities). On the other hand, in artificial seedlings, the mean was 10.9 and SD 0.6. Even in Kazumi, where the sample number was the same in both groups, SD was larger in artificial seedlings (0.6) than in wild fish (0.3). This means that the variation of abdominal vertebrae is larger in artificial seedlings. Accordingly, in artificial seedlings, the number of abdominal vertebrae is smaller and more variable than in wild fish.

The number of caudal vertebrae in wild fish ranged between 26.7 and 27.6 in the mean. The SD converged between 0.4 and 0.6, in spite of the differences of both localities and the number of samples. On the other hand, in artificial seedlings, the mean was 25.5 and SD 2.5. In artificial seedlings, the number of caudal vertebrae was two smaller and more variable than in wild fish.

The number of total vertebrae in wild fish ranged be-
between 37.3 and 38.7 in the mean, and between 0.4 to 0.6 in SD. In artificial seedlings, it was 36.4 in the mean and 2.5 in SD. In comparison of artificial seedlings with wild fish, the counts in the vertebrae were the same as those of the caudal vertebrae. Accordingly, the differences between wild fish and artificial seedlings in the mean and SD of the total number of vertebrae are considered to reflect directly to those of the caudal vertebrae.

**SHRINKAGE AND FUSION IN CENTRA**

The most typical symptom in bone anomaly observed in the vertebral column of *P. olivaceus* is that the neighboring centrums shrink and fuse each other (Fig. 5, middle, lower). In the artificial seedlings from Mie, the frequency of shrinkage and fusion in the centrums amounted to 63% (32 in 51 individuals). It showed a gentle peak around the fourth to seventh abdominal vertebra, while it was the lowest between the BOAP and the first abdominal vertebrae, at the anterior end of the vertebral column (Fig. 6). On the other hand, in wild fish from Tottori, shrinkage and fusion of abdominal vertebrae were not seen in any of 221 individuals.

The frequency of shrinkage and fusion in caudal vertebrae amounted to 74% (37 in 50 individuals) in the artificial seedlings from Mie. On the whole, the frequency presented three peaks: the gentle highest peak around the 10th to 12th caudal vertebrae, the second peak around the 18th to 19th and the third small peak around the 24th to 26th (Fig. 7). No fusion was seen between the 26th and 27th caudal (the urostyle) vertebrae, the fastest to be ossified. On the other hand, in the wild fish from Tottori, the frequency of fusion was only 4% (8 in 221 individuals). Though fusion was seen to lie scattered in the posterior caudal vertebrae, it composed a characteristic peak around the 25th and 26th caudal vertebrae. This peak seemed to correspond to the third peak seen in wild fish.

**DISCUSSION**

**DEVELOPMENTAL STAGE**

The developmental process of the axial skeleton in *P. olivaceus* was described following the developmental stages defined by Minami (1982). The formation of vertebrae proceeds intermittently from stage A, a stage soon after hatching, to stage I, a stage soon after settlement. In the formation, two remarkable phases, when ossification drastically proceeds, were also recognized. That is, stages C and F. The former is characterized by the formation of cartilages, such as neural and haemal arches. The latter is a phase when ossification drastically proceeds, regardless of origin. Accordingly, from the point of view in the developmental process of the vertebral column, the developmental stages of *P. olivaceus* were grouped into three periods, that is, A-B, C-E and F-I.
The gradation in early development of *P. olivaceus* was already ascertained with morphometric analysis, in which two points of drastic changes were found around 7 nun and 10 nun TL, respectively. The first point was situated between stages D and E, while the second between stages F and G. Paying attention to changes in external organs, the developmental process of *P. olivaceus* is to be grouped into three periods: A-D, E-F and G-I. Comparing the result in morphometry with that in development of the vertebral column, the staging does not always agree with the staging based upon changes in external organs. The shift of one developmental stage to the next always occurs earlier in the former stage than in the latter. However, it is obvious that changes in external organs, reflected in morphology, follow those in internal organs, e.g., the skeletal system. The structural changes in vertebrae supporting the body bring about the malformation of the external features. The points of changes in the early life history of *P. olivaceus*, especially feeding habit, are in accord with the staging based upon external organs (Hosoya 1991). Accordingly, the precedence of bone formation in the vertebral column to the formation of external organs might be regarded as a preadaptation to the upcoming stage.

Concerning cranial and caudal skeletons, in both skeletons, there is a discontinuous zone characterized by drastic ossification of membrane bone between stages D and E. Contrary to this, in the vertebral column, a drastic phase of ossification was present at stage F. Though it is difficult to explain the difference of phases in ossification between cranial and caudal skeletons, and vertebral column, it is partly explained by the functional property in each bone element. In bone formation of *P. major*, the life-support or feeding organ, swimming organ, and body-support organ were formed in order (Matsuoka 1987, Hosoya 1991). The cranial skeleton involves the basic bone elements related to life-support and feeding organs, e.g., gill arches and a gill apparatus, while the caudal skeleton is an important swimming organ, in charge of propulsion. It seems common in not only *P. major* but also the other marine fishes with larval and juvenile stages, that the formation of body-support organs is later than that of the cranial and caudal skeletons.

**CHARACTERISTICS OF ARTIFICIAL SEEDLINGS**

As one of the approaches to clarify the morphological characteristics of artificial seedlings, both wild fish and artificial seedlings of *P. olivaceus* were compared, concerning the frequency of centro fusion. As a result, in wild fish, fusion was hardly seen in other vertebrae, except for the 25th and 26th vertebrae. Contrary to this, in artificial seedlings, fusion was frequently seen. Especially in the seedlings from Mie, the frequency of fish with any fusion in the centrum was 74%. As the same tendency was also seen in the seedlings from Totori, centro fusion may be said to be characteristic of artificial seedlings. In fact, the stomach contents of a paddle crab, *Ovalipes punctatus*, captured during research on predators of released *P. olivaceus* by the Totori Prefectural Fisheries Experimental Station were analyzed, both fused abd onal and multi-fused vertebrae, not seen in wild fish, were observed together with such bone splinters as the urohyal, a lower jaw, and the hypurals, which certainly represent taxonomic characters of *P. olivaceus*. This evidence of predation on *P. olivaceus* with centro fusion suggests that centro fusion is a problem to be overcome in artificial production of healthy seedlings, which may also affect seedling survival upon release. Since centro fusion is not rare in wild larvae, elimination of such larvae by intense natural selection absent in hatcheries must occur. If artificial seedlings with centro fusion will be rapidly eliminated by natural selection upon release, then resource enhancement effects from release of such seedlings will be overestimated. Then, it becomes absolutely necessary to estimate the post-release survival rate of individuals with centro fusion empirically. As one of the practical methods for this purpose, in closed environments like salt pens, periodical estimation of the frequency of fish with centro fusion after being released might be considered.

**Bone formation and anomaly**

Artificial seedlings are characterized by central fusion, whose frequency shows one gentle peak in abdominal vertebrae and three peaks in caudal. The reason why the frequency of central fusion is high in artificial seedlings but low in wild fish is that the breeding environment of artificial seedlings is considered to deviate from the most optimal condition to control the morphogenesis of meristic characters, compared with the natural habitats of *P. olivaceus*. The factors inducing central fusion under the breeding environment are presumed to be: shortage of nutrient substance, water temperature, concentration of Ca²⁺, shortage of vitamin C, parasites, fish density and stress (Kitamura 1969, Matusato 1986). It is, however, difficult to specify the direct factors. Interestingly, among the peaks in the frequency of central fusion in artificial seedlings, only the third peak in the caudal vertebrae was seen in wild fish as well. This peak is present around the 25th and 26th caudal vertebrae, which are final formations in *P. olivaceus*. Though in the formation of axial skeleton, the genetically programmed fate in development might be considered to proceed, adjusted by environmental factors, the 25th and 26th caudal vertebrae are inferred to be the most sensitive region in development of *P. olivaceus*, in both artificial seedlings and wild fish. Body shrinkage is induced by either central shrinkage or shortage of an in-
terval between the neighboring centrum (Matsusato 1986). As each neighboring centrum is linked by a tendon, the 25th and 26th caudal vertebrae might be considered to be drawn closer and fused by the shrinkage of tendons before the completion of centrum ossification.

ACKNOWLEDGMENTS

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Further Advances Toward the Microbial Management in Closed Recirculating Production Systems of Marine Fish Larvae

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ABSTRACT

Management of the microbial species composition in closed culture systems is a viable strategy to enhance production of marine fish larvae. The procedure consists of eliminating the major sources of microbes through specific disinfection techniques and replacing the unwanted bacteria with a beneficial microflora. Disinfection methods were chosen to eliminate unwanted toxic residues and selection for resistance in the microflora. Selection of beneficial bacteria for larval culture was carried out as a two step procedure: (1) determining active nitrifying bacteria, and (2) evaluating either commercially available bacterial supplements or single bacterial strains found beneficial for the culture of diverse marine organisms, each supplied with the best nitrifiers as previously determined. Results to date are encouraging but one task remains and this is to develop rotifer cultures with known bacterial composition. Experiments with the marine fish Sciaenops ocellatus showed the feasibility of this management strategy to enhance fish production.

INTRODUCTION

Technological developments in marine fish culture are moving toward high density production systems. Large variability in larval survival and growth is recurrently observed among tanks stocked with larvae from a single batch of eggs. This variability in larval production needs to be reduced in order to make intensive fish production in closed systems a viable industry. The influence of the microflora on this variability has not been evaluated.

METHODS AND RESULTS

Variables were evaluated independently. However, the most successful method of production as determined at each step was incorporated into the procedure to test subsequent variables in order to build up a management strategy.

ELIMINATION OF BACTERIA OF UNKNOWN CHARACTERISTICS

WATER TREATMENT

Tanks with internal biological filters (Craig et al. 1990) were filled with 1.50 L tap water and treated with sodium hypochlorite (10 ppm Cl₂, final concentration). Following overnight aeration, tanks were drained rinsed copiously with tap water and filled with either raw seawater (control) or seawater filtered through 0.2 pm industrial cartridges. Seawater was aerated overnight before the addition of larvae.

CULTURE TECHNIQUES

Twelve hr after hatching, fish larvae were transferred to culture tanks at a density of 2,000 tank⁻¹. Three or four replicates were set per treatment. Beginning on the third day of culture, larvae were fed daily on washed rotifers, maintaining a prey density of 1 to 5 ml⁻¹. Artificial feeds were supplied to culture tanks with automatic feeders, at a rate of 0.4 to 1 g tank⁻¹ day⁻¹. After 10 days of culture, survival rates were calculated as:

Final number of fish/initial number (2,090) x 100.

Measurements were taken on 50 live, anesthetized fish from each tank, using a stereomicroscope, and a digitizing tablet and Sigma Scan software. Percentage survival and standard length were analyzed using one and two-way ANOVA and Tukey’s multiple range test (T-method; Sokal and Rohlf 1981) to determine the differences between treatments at the 0.05 level of probability. Larval growth was significantly higher in filtered seawater than in raw seawater (Tukey’s test, p < 0.05, Fig. 1).

EGG DISINFECTION

Fish eggs were treated with 3% hydrogen peroxide to obtain bacteria-free larvae (Douillet and Holt 1994). Eggs in control treatment were exposed to filtered seawater and manipulated as eggs treated with hydrogen peroxide. Larvae hatched from treated or non-treated eggs were cultured in filtered seawater and fed as previously described.
The experiment was run four times. Survival data was combined and showed a statistically significant improvement by disinfecting the eggs (Tukey’s test, p < 0.05; Fig. 2). Growth data was analyzed for each experimental run due to differences between experiments. Larvae from disinfected eggs were significantly larger than larvae from non-treated eggs only in experiment 1 (Tukey’s test, p < 0.05; Fig. 3).

**SELECTION OF BACTERIA BENEFICIAL FOR LARVAL CULTURE**

**NITRIFYING BACTERIA**

Bacteria-free larvae were cultured in filtered seawater and fed as described above. Tanks were covered with individual clear plastic bags to maintain independent microbial communities in each tank. Five different commercially available nitrifying bacterial mixtures were added independently to tanks at 250 ppm on the third day of culture. Control cultures did not receive any addition of nitrifying bacteria. Although no significant differences in larval survival or growth were detected between treatments after 10 days of culture, the rate of nitrification was significantly higher in tanks receiving the addition of nitrifiers “5,” a commercial blend of bacteria, than in all the other treatments (Tukey’s test, p < 0.05; Fig. 4).

**BACTERIAL SUPPLEMENTS**

Twelve bacteria strains and three commercial microbial additives were tested. Bacteria-free larvae were cultured in filtered seawater and fed as described above. Bacterial additives were added to the tanks as soon as they were filled with filtered seawater on day 1, and on days 4 and 7. Bacteria were added at a final concentration of **1x10^5** cells ml^-1. Nitrifiers “5” were added on the third day of culture to all tanks. No bacterial additives besides nitrifiers “5” were added to control cultures. There was an improvement in mean larval survival by the addition of a bacteria strain in experiments 1 and 4 (Fig. 5); however, because of large standard deviations, there was no statistical difference in survival between treatments. In the first three experiments, the addition of the probiotic reduced the coefficients of variation of larval survival between replicates compared to control cultures. In the fourth experiment, addition of the probiotic enhanced larval survival; however, this time the addition of the probiotic increased coefficients of variation of larval survival between replicate cultures, which was not consistent with previous experiments (Table 1).

**CONCLUSION**

Experiments with the marine fish *Sciaenops ocellatus* demonstrate the feasibility of applying a microbial...
management strategy. Complete larval mortalities frequently observed in some tanks prior to the use of microbial management were eliminated by following the methods described here. These microbial management techniques can be easily applied in commercial hatcheries.

The utilization of probiotics did not satisfy the requirement of consistent improvement in larval survival. Rotifers constitute a major source of unwanted bacteria in culture systems. Daily additions of large quantities of unwanted microbes contaminating the rotifers might have added to the variability in fish culture production and reduced the beneficial effects of probiotics which were only added every three days. Research in progress deals with methods of disinfecting and culturing rotifers with selected microbes so that no unwanted bacteria are added to larval culture systems. The integration of this last step will permit a more controlled composition of the microbial community in larval cultures and a more predictable fish production in closed systems.

LITERATURE CITED

Fig. 4. Final concentrations of \( \text{NH}_4, \text{NO}_2, \text{NO}_3 \) in 10-day-old cultures of red drum larvae either inoculated with different nitrifying bacteria or not receiving any addition of bacteria (control). Three replicate tanks were run per treatment. Columns significantly different (Tukey's test, \( p<0.05 \)) from other columns representing same form of nitrogen were identified with different letters.

Table 1. Coefficients of variation of larval fish survival in 10-day-old larval cultures either receiving or not (control) additions of probiotic.

<table>
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<th>Experiment</th>
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<th>Probiotic</th>
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Accumulation and Toxicity of Cadmium in Marine Fish

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ABSTRACT
Salinity affects the toxicity of several heavy metals in the aquatic biota, particularly cadmium. In this study the difference of physiological functions for osmotic regulation between freshwater and saltwater fish was applied to examine cadmium accumulation and its toxicity in marine fish. In the girella exposed to cadmium, the highest concentration of cadmium was found in the liver, followed by the kidney. The cadmium concentration in the gills was low. Cadmium accumulation in the gills of mummichog exposed to cadmium disproportionately decreased with increasing salinity, while the cadmium concentration in the intestine increased with salinity. After an intraperitoneal injection of cadmium bound to metallothionein to mummichog, the cadmium concentration in the kidney decreased with increasing salinity and the concentration in the hepatopancreas showed a tendency to decrease with salinity. Results from gel filtration of the gill cytosol of the exposed carp indicated that the gills were more likely to be harmfully affected by exposure to an acute level of cadmium than the intestine tissue. In red sea bream, results from gel filtration of tissue cytosol suggested that cadmium accumulated in the intestine at a high level and the maximum detoxification capacity in this tissue was reached earlier than in the gill tissue. The metallothionein induced in the red sea bream liver by cadmium and zinc was separated into two isoforms by anion exchange column. However, the ratios of two isoforms when induced by zinc were different from those induced by cadmium.

INTRODUCTION
In recent years, people have been concerned that marine pollution by various pollutants is slowly advancing in extensive areas all over the world. In classical toxicology, a frequently used measure for the toxicity of a pollutant is the concentration which kills 50% of a group of animals within a given time period (LC50). In modern times, there is a need to develop assays for the degree of toxicity resulting from sublethal exposure to contaminants in the environment, since there may be no clear outward signs of toxicity in chronically exposed organisms.

Additionally, there is a need to develop contaminant-specific assays of toxicity since organisms may be exposed to various potentially harmful contaminants in the environment. Research on sublethal effects of contaminants has been attempted to date (Passino 1984) and various investigators have reported on their particular techniques for measuring the effect of pollution (Szumski and Barton 1983). Unfortunately, there has been little agreement among investigators about which techniques are best for what and how they might be used for controlling marine pollution.

Heavy metals are among the most toxic and inevitable pollutants. There are several sources of heavy metal pollution, including mining industry, agricultural and silvicultural activities, waste disposal, and fossil fuel combustion. Cadmium is an extremely toxic element of continuing concern because its environmental levels have risen steadily with continued worldwide industrialization.

In order to understand better the impact of heavy metals, such as cadmium in aquatic organisms, it is important to understand the chemical and physiological processes that control their uptake, accumulation, storage and elimination. Although there have been many papers on cadmium accumulation and its toxicity to fish, most of them have dealt with freshwater fish. Perhaps it may be because the LC50 values of cadmium in saltwater fish are generally higher than those in freshwater fish. The marine environment may be deteriorating so that cadmium accumulation and its toxicity to marine fish must be further studied.

The aim of this study is to clarify the characteristics of cadmium accumulation and its toxicity in marine fish.
RESULTS AND DISCUSSION

DISTRIBUTION OF CADMIUM IN GIRELLA, GIRELLA PUNCTATA

Distribution of cadmium in the tissues of freshwater fish has been extensively studied (Nishihara et al. 1985). However, only limited data are available on cadmium accumulation in marine fish (Hilmy et al. 1985). Results from exposure of *girella, Girella punctata,* to Cd at 250 µg/L for up to 24 wk clearly showed that cadmium accumulates in the livers and kidneys of marine fish (Kuroshima 1987) (Fig. 1). It should be noted that this accumulation was observed in the liver even after the end of exposure. In cadmium-treated rainbow trout, increase in total amount of cadmium in the liver and kidney after the end of exposure has been reported (Kumada et al. 1980). Cadmium content in the kidney and intestines of mice administered this metal by subcutaneous injections increased even after dosing ceased (Nicholson et al. 1984). Cadmium once taken up in the body thus appeared to be hardly excreted but redistributed among tissues. Cadmium detected in the intestines of exposed *girellas* is possibly due to the intake of water to maintain the water balance. Maximum cadmium accumulation in the gills of fish in Cd at 250 µg/L was less than twice that in Cd at 25 µg/L (data are not shown), indicating the tissue does not have capacity to retain the metal.

CADMIUM ACCUMULATION IN THE MUMMICHOG, FUNDULUS HETEROCLITUS, ADAPTED TO VARIOUS SALINITIES

Adult males and juveniles of *mummichog* adapted to freshwater and 5%, 10%, 15%, 20%, 25%, 50%, 75% and 100% seawater (v/v) were exposed to Cd at 1 mg/L for 24 h (Kuroshima 1992a). The hardness of freshwater and 25% seawater used in this study was approximately 45 mg/L and 250 mg/L as CaCO₃, respectively. Cadmium concen-
trations in the whole body of juveniles, and in the hepato-
pancreas, kidney and gills of adult fish disproportionately
decreased with increasing salinity, ranging from freshwater
to 25% seawater and showed no further change at higher
salinity (Fig. 2a). Depression of cadmium accumulation
was most remarkable in the gills. Freshwater fish take up
most of the ions necessary for homeostasis from water via
the gills (Eddy 1982). Verboest at al. (1987) reported that
Cd\[^{2+}\] readily enters branchial epithelial cells of freshwater
fish, as does Cd\[^{2+}\] via La\[^{3+}\]- sensitive apical Ca\[^{2+}\] chan-
nels. Part and Svanberg (1985) proposed that gill perme-
ability for cadmium decreases with increase in the hard-
ness of water. The rapid decrease in cadmium accumula-
tion in the gill, hepatopancreas and kidney of mummichog
exposed in freshwater to 25% seawater was, thus, prob-
ably due to decrease in the active uptake of cadmium in
the gills. The cadmium concentration in the intestine in-
creased with salinity from freshwater to 100% seawater
(Fig. 2b). Marine fish swallow seawater and absorb water
together with monovalent ions from the intestine to com-
penstate for water loss from the body. The higher concen-
tration of cadmium in the intestine of mummichog adapted
to the higher salinities may possibly have been due to the
ingestion of water containing cadmium. Since water is
actively, but cadmium poorly, absorbed from the intestine,
cadmium dissolved in the water can be concentrated in
the intestinal tract. In mammals, cadmium orally adminis-
tered induces histopathological changes (Phillpotts 1986),
inhibition of enzyme activity (Kobayashi and Kimura
1985), and transport of nutrients (Itturi and Pena 1986) in
the small intestine. The intestinal function of seawater fish
may possibly be damaged during exposure to cadmium.

Data from mammal experiments generally demonstrate
cadmium bound to metallothionein to be less effectively
trapped by the liver and mostly taken up by the kidney
(Suzuki 1984). Results from the administration of cadmium
bound to metallothionein by an intraperitoneal injection
are shown in Fig. 2c. The cadmium concentration in the
kidney decreased with increasing salinity and at salinities

\[\text{Cd concentration (\text{\textmu}g./ml.)}\]

\[\text{Fraction number}\]

\[\text{Fraction number}\]

\[\text{Fraction number}\]

\[\text{Fraction number}\]
of 75% and 100% seawater were significantly lower than those in freshwater. The cadmium concentration in the hepatopancreas showed a tendency to decrease with salinity. The ratio of cadmium concentration in the kidney to that in the hepatopancreas in each fish decreased with salinity. In the kidney of freshwater fish, essential electrolytes, glucose and vitamins are selectively reabsorbed from urine at the proximal tubules, while the main function of the kidney of marine fish is excretion of divalent cations. The function of the kidney to maintain the proper osmotic conditions in the body may, thus, possibly be related to the accumulation of cadmium bound to metallothionein in the kidney.

CADMIUM CYTOSOLIC PARTITIONING IN THE TISSUES OF CARP AND RED SEA BREAM

The carp, *Cyprinus carpio*, and red sea bream, *Pagrus major*, were exposed to Cd at 0.3 mg/L and 10 mg/L, respectively, for 96 h (Kuroshima 1992b). The cytosols of the gills and intestine were separated into fractions according to molecular weight using Sephadex G-75 gel chromatography. Cadmium in tissue cytosolic extracts were separated into a high molecular weight (HMW, >20,000 Da) pool, a medium molecular weight (MMW, 3,000-20,000 Da) pool and a low molecular weight (LMW, <3,000 Da) pool. The term Da denotes a unit for molecular weight of protein. The cadmium in the HMW pool containing enzymes and that in the MMW pool containing metallothionein was regarded as being potentially toxic and detoxified, respectively. Results from Sephadex G-75 gel filtration of the gill cytosol of the exposed carp indicate that the gills are more likely to be harmfully affected by exposure to an acute level of cadmium than the intestine tissue (Fig. 3a, b). In red sea bream, results from gel filtration of tissue cytosols indicate that cadmium accumulated in the intestine at a high level and the maximum detoxification capacity in the intestine was reached earlier than in the gills (Fig. 3c, d). It is reported that the intestine of scorpionfish may be the most highly influenced tissue on exposure to high cadmium concentrations (Brown et al. 1990).

METALLCYTHIONEIN INDUCED BY CADMIUM AND ZINC IN THE LIVER OF RED SEA BREAM

Metallothioneins are low molecular weight metalloproteins in the liver, kidney, and other tissues of various species including aquatic organisms (Unger et al. 1991). They are characterized by unique amino acid compositions with a very high cystein content and the absence of aromatic amino acids (Fowler et al. 1987). Since metals like cadmium and zinc induce metallothioneins and bind to them via the sulphydryl group of cysteine residues, they may possibly be involved in protection against cadmium toxicity and homeostasis of zinc and copper (Brady 1982). Recent studies have shown hormones (Hylland et al. 1989), cytokines (Schroeder and Cousins 1990), and various forms of chemical and physiological stress (Kagi and Schaffer 1988) to induce metallothioneins and the expression of some genes to be under separate control and possibly to have different biological purposes (Sadhu and Gedamu 1988). The metallothionein induced in the liver of red sea bream by cadmium and zinc was separated into two isoforms by anion exchange column (Kuroshima 1995) (Fig. 4a). Following cadmium treatment, zinc was much higher in MTII than in MTI, where MTII and MTI denote isoforms of metallothionein. In metallothionein induced by zinc treatment, there were about equal amounts of zinc in both isoforms of metallothionein (Fig. 4b). These results suggest that the isoforms of metallothionein may have different biological functions.

CONCLUSION

Generally, when fish are exposed to pollutants, the organ or tissue most susceptible to adverse effects is the gills. This theory is based on results of experiments showing that gills are the main uptake site of many pollutants in
freshwater fish. However, the cadmium concentration in gills of girella exposed to the metal was low, and results from the examination of cadmium cytosolic partitioning in the gill tissue of red sea bream suggested that gills were not the most susceptible tissue. This discrepancy is considered to be partly due to the difference of osmoregulatory functions. The intestine is also an important organ for osmoregulation of salt fish. The relative sensitivity of the intestine to cadmium exposure is of particular interest.

The liver and kidney are usually considered to be the principal sites of chronic cadmium toxicity. The ratio of cadmium concentration in the kidney to that in the hepato-pancreas of the mummichog decreased with salinity tier and an intraperitoneal injection of metallothionein. This result indicates that the ability to trap the cadmium bound to metallothionein in plasma by kidney is lower in saltwater fish than in carp. Therefore, the liver in saltwater fish may suffer chronic cadmium toxicity more severely than that in freshwater fish.

Metallothionein is thought to be involved in detoxification of certain heavy metals, such as cadmium, because of the avidity with which they bind to such metals. In addition, metallothionein may play an important role in the homeostatic control of zinc metabolism. Although it has been known that metallothionein is induced in fish, the information on this protein in saltwater fish is limited. Metallothionein in tissues of red sea bream was found to be present in two isoforms.

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THE LIVER AND KIDNEY ARE USUALLY CONSIDERED TO BE THE PRINCIPAL SITES OF CHRONIC CADMIUM TOXICITY. THE RATIO OF CADMIUM CONCENTRATION IN THE KIDNEY TO THAT IN THE HEPATO-PANCREAS OF THE MUMMICHOG DECREASED WITH SALINITY TIER AND AN INTRAPERITONEAL INJECTION OF METALLOTHIONEIN. THIS RESULT INDICATES THAT THE ABILITY TO TRAP THE CADMIUM BOUND TO METALLOTHIONEIN IN PLASMA BY KIDNEY IS LOWER IN SALTWATER FISH THAN IN CARP. THEREFORE, THE LIVER IN SALTWATER FISH MAY SUFFER CHRONIC CADMIUM TOXICITY MORE SEVERELY THAN THAT IN FRESHWATER FISH.

METALLOTHIONEIN IS THOUGHT TO BE INVOLVED IN DETOXIFICATION OF CERTAIN HEAVY METALS, SUCH AS CADMIUM, BECAUSE OF THE AVIDITY WITH WHICH THEY BIND TO SUCH METALS. IN ADDITION, METALLOTHIONEIN MAY PLAY AN IMPORTANT ROLE IN THE HOMEOSTATIC CONTROL OF ZINC METABOLISM. ALTHOUGH IT HAS BEEN KNOWN THAT METALLOTHIONEIN IS INDUCED IN FISH, THE INFORMATION ON THIS PROTEIN IN SALTWATER FISH IS LIMITED. METALLOTHIONEIN IN TISSUES OF RED SEA BREAM WAS FOUND TO BE PRESENT IN TWO ISOFORMS.
