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Recruitment studies:
Manual on precision and accuracy of tools

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Contents

Recruitment studies: Manual on precision and accuracy of tools

This manual is one of the results of the project Precision and Accuracy of Tools in Recruitment Studies (PARS), financed by the EU (FAIR-CT96–1371). The project PARS is concerned with improving the methodologies used in investigations and the monitoring of the early life stages of fish larvae, especially herring and sardine. These are important in both stock assessments and strategic research intended to improve predictive capability. The project deals with precision and accuracy issues in two categories of measurements, which together encompass most of the data that are routinely required from samples of early life stages of fish:

- the growth and condition of individuals;
- the origin of individuals.

Quantitative evaluation of the growth rate and condition of larvae in relation to environmental conditions at the time of capture is an essential prerequisite for predictive assessment of survival potential. In particular, the identification of starving or sub-optimally growing larvae in the population is a powerful method for evaluating survival probability. Both biochemical and otolith microstructure methodologies offer the prospect of identifying individuals at risk. However, the understanding of the underlying physiology of somatic and otolith growth and development is not sufficiently advanced; therefore, it does not allow a high degree of accuracy. In particular, decoupling of otolith and somatic growth under some circumstances is recognized but not understood.

Determining the origin of early life stages is important for a number of applications. Origin in this context refers to the time and location at which a specimen was hatched, and the history of an individual's growth rate and the environment between hatching and capture. A combination of otolith microstructure and chemical analyses provides the tools necessary for this study. The project evaluates the discriminatory power of these methods using controlled mesocosm and laboratory experiments and tests their precision by reference to field-caught material.

This manual is an important step for standardizing techniques used in recruitment studies and will be beneficial to anyone working in the field of fish recruitment in order to increase the knowledge of fish stocks and their management.

1 OTOLITH MICROSTRUCTURE

1.1 Introduction

The discovery of daily growth increments in otoliths of fish larvae and fry (Pannella, 1971) opened the way to study, in detail, the life history of fish larvae and juveniles. By determining the age in days, it is possible to calculate an individual's growth rate, if length and weight relationships are available.

Otoliths serve as a permanent record of the life history of an individual fish. For most species, the formation of daily growth rings starts at the end of the yolk-sac stage or at the time eyes become pigmented. The exact time of formation of the first increment varies among species. After the first ring around the nucleus, concentric rings are formed that in most cases are believed to be daily (Campana and Neilson, 1985; Jones, 1986). The distance between the rings is influenced by food uptake, temperature, and other environmental conditions. The distance between the rings expresses the daily growth of the individual, while the number of rings indicates its age in days. More information on otoliths and growth rates can be found in Secor *et al*. (1995) and Fossum *et al*. (2000).

1.2 Sampling and Preservation

In this study, the larvae and juvenile herring were sampled in the laboratory, in mesocosms, or at sea. The larvae or juveniles should be preserved quickly after sampling either in 96 % ethanol or in liquid nitrogen (see Section 2.2 for more details). For samples stored in ethanol, the ethanol should be changed once after about 24 hours. To avoid the ethanol turning to acid, and thereby destroying the otoliths, it is recommended to buffer the ethanol with Tris (hydroxymethyl) aminomethane, 99.0–99.5 %, saturated solution, 40 %. This saturated solution is mixed with the ethanol in the proportion 6.6 ml to 1 l.

Mounting of otoliths

The length of the larvae/juveniles should be measured to the nearest 1.0 mm standard length. The largest pair of otoliths, sagittae, is removed and mounted on a glass plate as shown in Figure 1. For mounting use Cytoseal, Pro-Texx, or clear nail polish (Sally Hansen ®: "Hard as Nails with Nylon").

Figure 1. Mounting of otoliths on a glass plate.
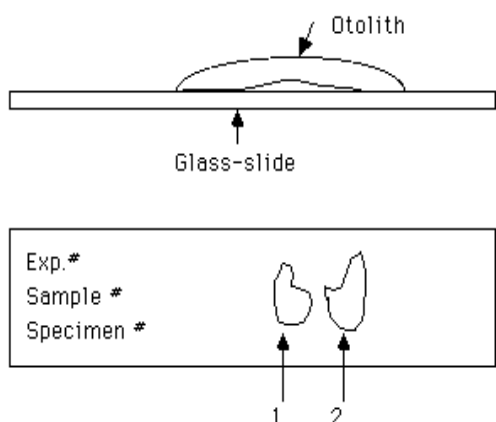
<u>Treatment of otoliths for reading</u>

Otoliths of, e.g., clupeoid juveniles over 30 mm in length may be too thick to allow the passage of enough light, so that the reader (operator) will be unable to see all the growth increments. This necessitates the removal of material from the otoliths to expose growth rings. This can be done either by using acid (5–10 % HCl) or fine sand paper (0.3 µm or 30 µm). When using acid, the parts of the otoliths not to be treated can be masked with lens oil or a mounting medium.

1.3   Age Determination

The age is calculated according to the formula of Methot (1981):

$$\sum_{i=0}^{n} \frac{r_i - r_{i-1}}{G(r_i)}$$

$G(r_i)$ = Average increment size between r_{i-1} and r_i;
r_0 = Otolith radius to first increment (hatch check);
r_n = Total radius (The distance: Focus to Margin);
$G(r_0)$ = First increment size (about 0.8 µm);
$G(r_n)$ = $G(r_{n-1})$ if $G(r_n)$ is not measured.

The method is based on the assumption that the reader can read a portion of the otoliths, and that there may be a part of the otolith where increments cannot be observed. In these parts of the otolith, the expected number of increments is calculated from the mean increment size of the adjacent areas. A sketch of the method is given in Figure 2. The time of formation of the first daily increment will vary from species to species.

Figure 2. A sketch of the ageing method. Edge of focus = hatching check.
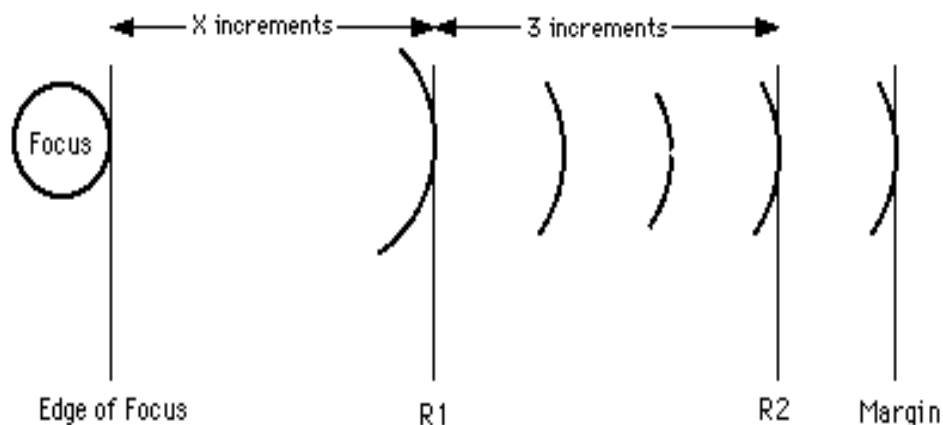
1.4   Accuracy of Age Estimate

Studies have shown that the first zonal increment in herring larvae is formed at the time of yolk-sac absorption (EYS) (Geffen, 1982; Lough *et al*., 1982; Moksness, 1992b; Høie *et al*., 1999). Depending on egg size and the temperature, the age of EYS for spring-spawning Clyde herring larvae has been measured to be between six and ten days in independent rearing studies (Geffen, 1982). It is therefore necessary to apply a correction factor to the estimated age of the larvae. A correction factor of eight days gave the most accurate age estimates in our experiment with

spring-spawning Clyde herring and Norwegian spring-spawning herring. This is somewhat lower than the ten days used by Andersen and Moksness (1988), Moksness (1992a), and Stenevik *et al.* (1996). However, in our experiment ten days gave a larger biased age estimate of the larvae raised under high food conditions, especially for the youngest age groups.

With regard to sardine larvae, Alemany and Alvarez (1994) reported increment formation in the embryonic stages of sardine. From rearing experiments carried out on sardine, the size of the otolith radius at hatch ranged from 5–6 µm. At this point, a rather clear check is formed in the otoliths of sardine larvae. Thereon, very fine (from 0.7–0.8 µm) increments are discernible in agreement with the observations of Alemany and Alvarez (1994). A series of very fine increments (around five) are estimated before these become wider and sharper visually. At this point another check is visible, which some researchers attribute to the first-feeding check. All these described features of sardine otoliths are clearer with the size of the sampled larvae. Post-larval stages, from 15 mm on, have sharper and clearer increments than the early larval stages. Therefore, no correction factor in regard to age estimates was applied to this species.

Photographic Plates 1 and 2 exemplify the description mentioned above.

Plate 1.

Day 1 sagitta of sardine larva (1000 magnification)

Day 3 sagitta of sardine larva (1000 magnification)

Sardine larva 5 days old in which the otoliths are clearly visible under polarized light.
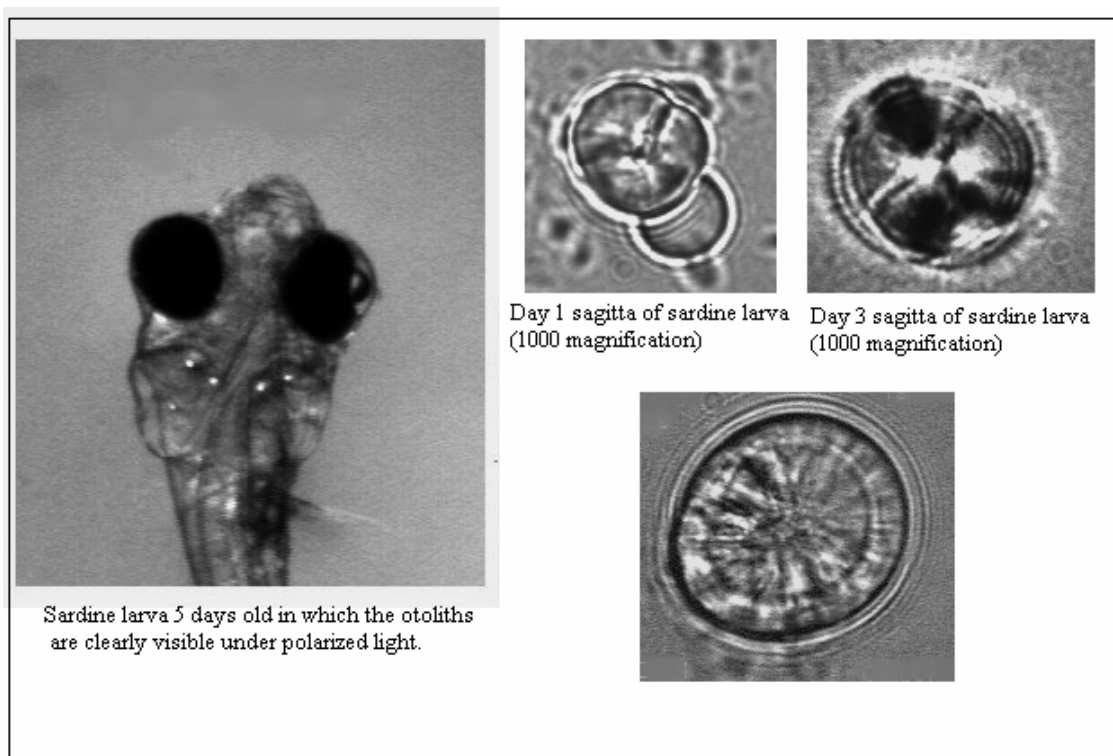
Plate 2.

Sagitta of sardine larva visualized at 1,000 magnification in which the inner arrow indicates edge of focus and the outer one, the first-feeding check.

The accuracy of the age estimates of larvae raised under low food conditions was found to be extremely low in this study. None of the larvae obtained an acceptable age estimate (within ±10 percent of actual age). This was caused by a large discrepancy between the number of increments and the age of the larvae. This discrepancy ranged from 14 days to 28 days in three- and six-week-old larvae, respectively, kept at low food density. Several authors have reported similar discrepancies in slow-growing larvae: 20–30 days (Moksness *et al.*, 1987), 17 days (Lough *et al.*, 1982), 15–20 days (Campana *et al.*, 1987), 15–17 days (Messieh *et al.*, 1987), and more than 40 days in some treatments (Geffen, 1982).

The discrepancy between the number of increments and the age of the larvae was considerably larger than expected if the first increment was to be formed 6–10 days after hatching. This indicates non-daily increment deposition in herring larvae reared at low food density. Non-daily increment deposition in slow-growing herring larvae has previously been reported by Geffen (1982), Lough *et al.* (1982), McGurk (1984), Moksness *et al.* (1987), and Folkvord *et al.* (2000).

Limitations in light microscope resolution

Limitations of the light microscope (LM) may explain observation of non-daily increment formation in larvae with low growth. The theoretical resolution[1] of a light microscope is 0.20

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[1] Resolution is defined here as the minimum distance between two structures consistent with the two structures remaining visually discernable.

µm (Campana, 1992; Neilson, 1992). However, the resolution is affected by both the quality of the equipment and the skill of the operator (Campana, 1992). According to Brothers (1987) and Neilson (1992), the practical resolution limit is closer to 0.5–1.0 µm. In our experiment, the average otolith growth was less than 0.6 µm/day in almost all larvae reared at low food densities (Figure 3). If we assume daily increment deposition, a large proportion of the increments would then be too narrow to be recognized in a light microscope. One would expect to observe these narrow increments using SEM (Scanning Electron Microscopy), which has the advantage of providing a much higher resolving power (Brothers, 1987). Several experiments have shown discrepancies in the increment counts obtained using light microscopy and SEM (Neilson, 1992). Using SEM, Campana and Moksness (1991) observed narrow increments (0.2–0.3 µm) in the area close to the hatch check. These increments were not observed using a light microscope (Moksness, 1992b). These results suggest that the non-daily increment formation observed in our experiment can to some extent be due to limitations in the resolution of the light microscope.

Figure 3. Otolith growth (µm/day) plotted against deviation of age estimates (%) from actual age. □ = Low prey density; ♦ = High prey density. The figure presents data from all herring larvae in the experiment. It is assumed that the first increment is laid down eight days after hatching. Points on the solid line have an estimated age equal to the actual age. Within the broken lines, the age is under-/over-estimated by less than 10 percent. Outside the broken lines, the age of the larvae is under-/over-estimated by more than 10 percent.
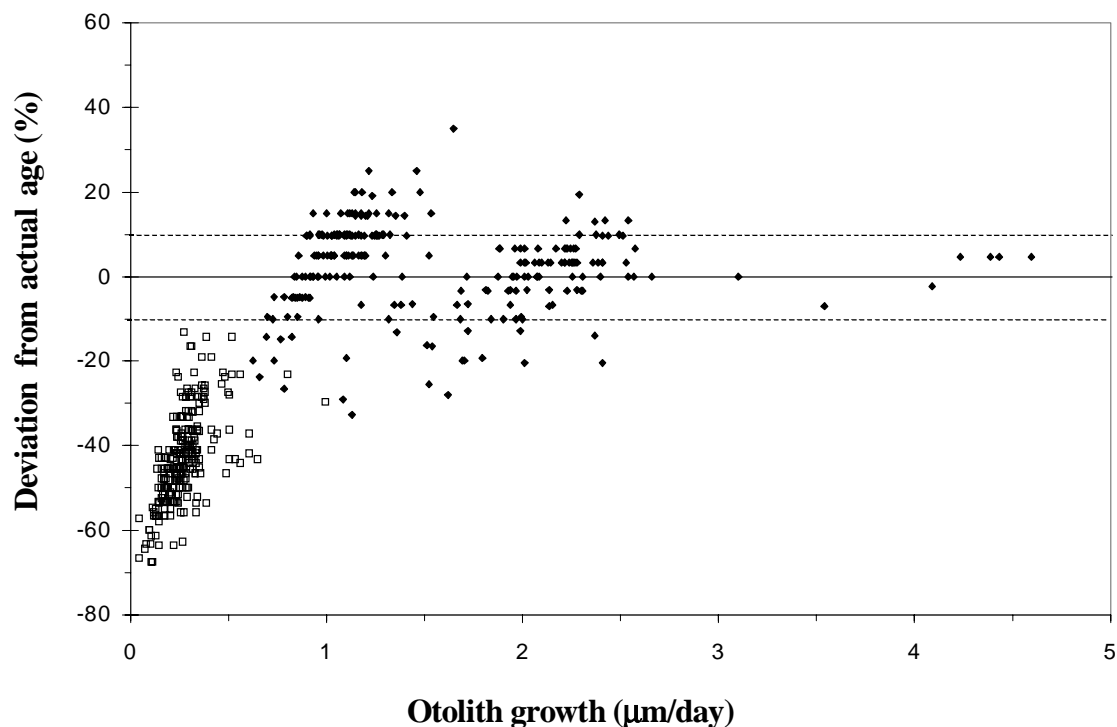
The accuracy of the age estimates was found to be dependent on the otolith growth for growth rates less than 0.8 µm/day (Figure 3). Limitations in the resolution of the light microscope may explain why the accuracy was found to be dependent on the otolith growth in these larvae. For average increment widths less than 0.8 µm/day, a larger proportion of the increments will be too narrow to be observed in a light microscope.

<u>High food density</u>

The accuracy of the age estimates was considerably higher in the fast-growing larvae than in slow-growing larvae. In the two youngest age groups (three and four weeks), 75 % and 85 % of the larvae had an acceptable age estimate (within ±10 percent of actual age). The discrepancy between the number of increments and the age was seven and eight days, respectively. This is consistent with the estimates of six and ten days discussed by Geffen (1982), and indicates that increments are formed on a daily basis from the time of yolk-sac absorption (EYS) in fast-growing three- and four-week-old larvae. Similar results have been reported by Moksness and Wespestad (1989), who observed daily increments in Pacific herring (*Clupea pallasii*) from the end of the yolk-sac stage (age 8 days). Daily increment formation in spring-spawning herring with normal growth has been reported by Campana and Moksness (1991) and Moksness (1992b).

The accuracy of the age estimates in six-week-old larvae, reared at high food levels, was unexpectedly low. An acceptable age estimate was found in only 57 percent of the larvae in this group. The low accuracy was probably caused by a low growth rate in several larvae in this group. Closer examination showed that the otolith growth was significantly lower in the larvae with underestimated age, compared with larvae with an acceptable age estimate.

Alternatively, the low accuracy may be due to a lack of preparation. In smaller otoliths, mounting and/or clearing will provide sufficient resolution of microstructure (Secor *et al.,* 1992). Polishing of large otoliths will improve the resolution of narrow increments around the nucleus (Campana and Moksness, 1991). When the diameter of the otoliths exceeds 50 µm, polishing is mandatory (Campana *et al.*, 1987). Thus, otoliths of both four- and six-week-old larvae, reared at high food levels, should have been polished before reading. However, parts of the otolith may break when polishing (Neilson, 1992), and overgrinding may cause subdaily increments to become quite prominent. Further, polishing in an oblique plane represents an additional source of bias since the microstructure will then be obscured.

<u>Average increment width</u>

This project has confirmed that the accuracy of the age estimates was poor in slow-growing herring larvae. Thus, if the slow-growing larvae are not identified, the age estimates will be heavily biased. It is therefore important to investigate the possibility of identifying the slow-growing larvae from the information on the number of increment zones.

It can be seen from Figure 4 that it is not possible to distinguish three-week fast-growing larvae from slow-growing larvae. In all of these groups, the average increment width was less than 1.1 µm. Therefore, the age estimates of these larvae are highly uncertain if the rate of growth is not known. In herring larvae with an average increment width somewhat higher than 1.1 µm, the growth is most likely normal. Appropriate methods for identifying slow-growing larvae could be either the RNA/DNA ratio (Clemmesen, 1989) or the fatty acid composition (review by Bulow, 1987). In a study of autumn-spawning North Sea herring, Folkvord *et al.* (1996) found that the RNA/DNA ratio was significantly correlated with other growth measures. It was concluded that the use of RNA/DNA ratios and their derived growth indices provided useful measures of larval growth.

Figure 4. Average increment width (± S.D.) in the six groups of herring larvae. The average increment width is calculated using the following equation:
(Radius (µm) – distance from nucleus to first increment (µm)/no. increments read).
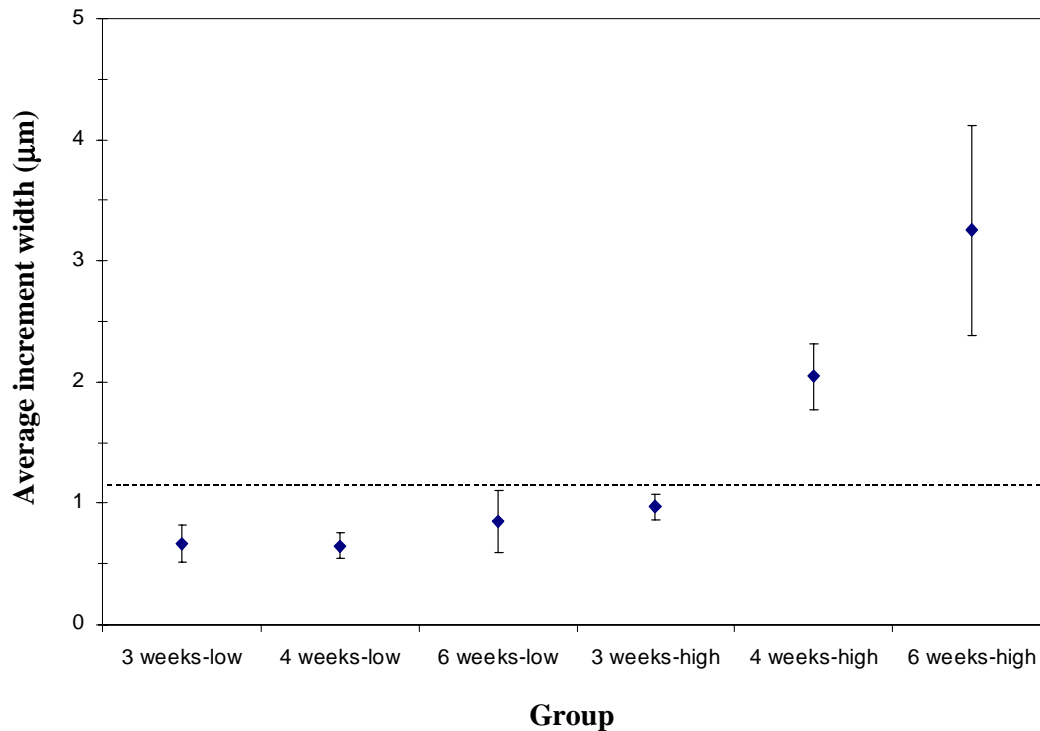
1.5   Conclusions

The results of this study show that the otolith microstructure provides useful information for age determination of herring larvae with normal growth. However, the experiments also reveal the importance of being cautious when aging young and/or slow-growing larvae. Limited resolution of the light microscope, causing several increments to be invisible, is considered to be the main reason for the low accuracy found in slow-growing larvae. Limited resolution of the light microscope may also explain why the accuracy is so dependent on the otolith growth when it is less than 0.8 µm/day. It is concluded that the average increment width of the otolith provides useful information to identify larvae in danger of receiving highly biased age estimates.

1.6   References

Alemany, F., and Alvarez, F. 1994. Formation of initial daily increments in sagittal otoliths of reared and wild *Sardina pilchardus* yolk-sac larvae. Marine Biology, 121: 35–39.

Andersen, T., and Moksness, E. 1988. Manual for reading daily increments by the use of a computer program. Flødevigen Meldinger, 4: 1–37.

Brothers, E.B. 1987. Methodological approaches to the examination of otoliths in aging studies. *In* The Age and Growth of Fish, pp. 319–330. Ed. by R.C. Summerfelt and G.E. Hall. Iowa State University Press, Ames, Iowa, USA.

Bulow, F.J. 1987. RNA-DNA ratios as indicators of growth in fish. *In* The Age and Growth of Fish, pp. 45–64. Ed. by R.C. Summerfelt and G.E. Hall. Iowa State University Press, Ames, Iowa, USA.

Campana, S.E. 1992. Measurement and interpretation of the microstructure of fish otoliths. *In* Otolith microstructure examination and analysis, pp. 59–71. Ed. by D.K. Stevenson and S.E. Campana. Canadian Special Publication on Fisheries and Aquatic Sciences, 117.

Campana, S.E., Gagné, J.A., and Munro, J. 1987. Otolith microstructure of larval herring (*Clupea harengus*): Image or reality? Canadian Journal of Fisheries and Aquatic Sciences, 44: 1922–1929.

Campana, S.E., and Moksness, E. 1991. Accuracy and precision of age and hatch date estimates from otolith microstructure examination. ICES Journal of Marine Science, 48: 303–316.

Campana, S.E., and Neilson, J.D. 1985. Microstructure of fish otoliths. Canadian Journal of Fisheries and Aquatic Sciences, 42: 1014–1032.

Clemmesen, C.M. 1989. RNA/DNA ratios of laboratory-reared and wild herring larvae determined with a highly sensitive fluorescence method. Journal of Fish Biology, 35: 331–333.

Folkvord, A., Blom, G., Johannessen, A., and Moksness, E. 2000. Growth dependent age estimation in herring (*Clupea harengus* L.) larvae. Fisheries Research, 46: 91–103.

Folkvord, A., Ystanes, L., Johannessen, A., and Moksness, E. 1996. RNA:DNA ratios and growth of herring (*Clupea harengus* L.) larvae reared in mesocosms. Marine Biology, 126: 591–602.

Fossum, P., Kalish, J., and Moksness, E. 2000. Proceedings from 2nd International Symposium on Fish Otolith Research and Application, Bergen, Norway, 20–25 June 1998. Special Issue of Fisheries Research, 46: 1–374.

Geffen, A.J. 1982. Otolith ring deposition in relation to growth rate in herring (*Clupea harengus*) and turbot (*Scophthalmus maximus*) larvae. Marine Biology, 71: 317–326.

Høie, H., Folkvord, A., and Johannessen, A. 1999. Maternal, paternal and temperature effects on otolith size of young herring (*Clupea harengus* L.) larvae. Journal of Experimental Marine Biology and Ecology, 234: 167–184.

Jones, C. 1986. Determining age of larval fish with the otolith increment technique. Fishery Bulletin U.S., 84: 91–103.

Lough, R.G., Pennington, M., Bolz, G.R., and Rosenberg, A.A. 1982. Age and growth of larval Atlantic herring, *Clupea harengus* L., in the Gulf of Maine-Georges Bank region based on otolith growth increments. Fishery Bulletin U.S., 80: 187–199.

McGurk, M.D. 1984. Ring deposition in the otoliths of larval Pacific herring, *Clupea harengus pallasi*. Fishery Bulletin. U.S., 82: 113–120.

Messieh, S.N., Moore, D.S., and Rubec, P. 1987. Estimation of age and growth of larval Atlantic herring as inferred from examinations of daily growth increments of otoliths. *In* The Age and Growth of Fish, pp. 433–442. Ed. by R.C. Summerfelt and G.E. Hall. Iowa State University Press, Ames, Iowa, USA.

Methot, R.D. 1981. Growth rates and age distributions of larvae and juvenile Northern Anchovy, *Engraulis mordax*, with references on larval survival. Doctor Thesis, University of California, San Diego. 209 pp.

Moksness, E. 1992a. Differences in otolith microstructure and body growth rate of North Sea herring (*Clupea harengus* L.) larvae in the period 1987–1989. ICES Journal of Marine Science, 49: 223–230.

Moksness, E. 1992b. Validation of daily increments in the otolith microstructure of Norwegian spring-spawning herring (*Clupea harengus* L.). ICES Journal of Marine Science, 49: 231–235.

Moksness, E., Butler, J., and Radtke, R.L. 1987. Estimation of age and growth rate in Norwegian spring spawning herring (*Clupea harengus* L.) larvae and juveniles. Sarsia, 72: 341–342.

Moksness, E., and Wespestad, V. 1989. Ageing and back-calculating growth rates of Pacific herring, *Clupea pallasii*, larvae by reading daily otolith increments. Fishery Bulletin U.S., 87: 509–513.

Neilson, J.D. 1992. Sources of error in otolith microstructure examination. *In* Otolith Microstructure Examination and Analysis, pp. 115–125. Ed. by D.K. Stevenson and S.E. Campana. Canadian Special Publication on Fisheries and Aquatic Sciences, 117.

Pannella, G. 1971. Fish otoliths: daily growth layers and periodical patterns. Science, 173: 1124–1127.

Secor, D.H., Dean, J.M., and Laban, E.H. 1992. Otolith removal and preparation for microstructural examination. *In* Otolith Microstructure Examination and Analysis, pp. 19–57. Ed. by D.K. Stevenson and S.E. Campana. Canadian Special Publication on Fisheries and Aquatic Sciences, 117.

Secor, D.H., Dean, J.M., and Campana, S.E. (eds.). 1995. Recent Developments in Fish Otolith Research. Belle W. Baruch Institute for Marine Biology and Coastal Research, University of South Carolina Press, Columbia, South Carolina, USA.

Stenevik, E.K., Fossum, P., Johannessen, A., and Folkvord, A. 1996. Identification of Norwegian spring spawning herring (*Clupea harengus* L.) larvae from spawning grounds off western Norway applying otolith microstructure analysis. Sarsia, 80: 285–292.

2 LARVAL CONDITION (RNA/DNA RATIO)

2.1 Introduction

Recruitment variability is considered to be determined by the survival during the early life history stages. It is accepted that starvation and predation, or the interaction of both, are the main factors of mortality during the larval stages (Houde, 1987). Thus, starvation weakens larvae, decreasing their capacity to avoid attack by predators and, hence, increasing their mortality by predation (Purcell *et al.*, 1987). On the other hand, starvation leads to slower growth rates (Buckley, 1982, 1984), and larvae spend more time in length classes that are more vulnerable to predators (Folkvord and Hunter, 1986; Rice *et al.*, 1993). Therefore, the evaluation of the nutritional condition of marine fish larvae may have a great importance for a

better understanding of the processes affecting survival during the early life stages. The nutritional condition of fish larvae can be determined by histological, morphometric, and biochemical methods (enzymatic activity, RNA/DNA ratios, and lipid content). Of all these techniques, nucleic acid determination has undergone a great development in recent years (for reviews see Ferron and Leggett, 1994; Bergeron, 1997; Buckley *et al.*, 2000).

The use of the RNA/DNA ratio as an indicator of fish larval growth and condition is based on the assumptions decribed here. The amount of DNA, the carrier of genetic information, is considered constant in somatic tissues, and is directly related to the numbers of cells of an individual. In fact, the relative DNA content (DNA/dry weight) has been used as an estimate of cell size (Bulow, 1987). However, the amount of RNA in cells is directly proportional to the protein synthesis rate. Thereby, the ratio RNA/DNA is an index of the cell's metabolic rate and has been proven to be a useful indicator of nutritional condition and growth rate in fish larvae (Buckley, 1984; Clemmesen, 1994; Westerman and Holt, 1994). Well-fed larvae and fast-growing larvae have higher RNA/DNA ratios and wider daily increment deposition than starving larvae (Wright and Martin, 1985; Hovenkamp, 1990; Hovenkamp and Witte, 1991; Clemmesen and Doan, 1996; García *et al.*, 1998; Ramirez *et al.*, 2001). Therefore, the joint study of larval otolith microstructure and larval condition biochemical indices is a promising methodology to research processes affecting recruitment variability. In several fish species, RNA/DNA ratios have been successfully related with food density and somatic growth (Buckley, 1984; Clemmesen, 1994; Rooker and Holt, 1996). However, other condition indices such as protein/DNA and RNA/protein ratios show less variability than RNA/DNA ratio, and several authors have found that they are better indicators of nutritional state and growth rate (Bergeron *et al.*, 1991; Richard *et al.*, 1991; Foster *et al.*, 1992). This high variability of the RNA/DNA ratio has called into question the reliability and accuracy of certain measurement protocols.

This section summarizes the results of several intercalibration exercises carried out within the PARS project. The partners involved in these intercalibration exercises were:

Partner 2: Department of Fisheries and Marine Biology, University of Bergen;

Partner 4: Spanish Institute of Oceanography, Oceanographic Centre of Malaga;

Partner 5: Institut für Meereskunde an der Univesität Kiel.

2.2 Sampling of Fish Larvae

The study of wild larvae involves sampling at sea. In order to study larval condition, some considerations during sampling have to be taken into account. The sampling of fish larvae should always be carried out at the same time of the day. This is due to a diel variation of the RNA content. Several authors have reported diel variations in RNA content and in the RNA/DNA ratio of fish larvae (Bergeron, 1997; Chícharo *et al.*, 1998). Tows should be short in time in order to avoid the degradation of larval tissues. For example, for superficial tows, tows of about five minutes' duration are recommended.

If tows are longer, larvae can lose their eyes and suffer breakage of tissues. Tows should be carried out with a boat speed of less than 2 knots; tows faster than this can damage the larvae. In order to avoid contamination of larvae, vials, and dishes by RNase from sweating of the technician's hands, we recommend the use of gloves during the sorting and manipulation of larvae.

Steps during sampling are:

1) When nets come on board, rinse the net gently with sea water;

2) Transfer content of the collectors into a bucket with cooled sea water;

3) Take the bucket to the dry lab, and transfer aliquots of its content into a large glass dish;

4) Larvae must be sorted quickly. Larvae are placed in glass dishes filled with filtered sea water and put into freezer packs;

5) Species must be identified under a binocular microscope. The larvae must be manipulated gently; do not use forceps because they can damage the larvae. The use of a fine paintbrush made of soft hair is recommended. During the process of sorting and identification, larvae should be kept in a bath of ice;

6) After sorting and identification, larvae should be kept in suitable vials labelled with a code. Cryogenic vials are recommended. Other kinds of plastic vials can break during the process of freezing and defrosting. In order to reduce the shrinkage of larvae and to facilitate the extraction of larvae from the vial without manipulation, the cryogenic vials should contain 0.45 µm filtered sea water;

7) Finally, larvae should be preserved in liquid nitrogen at −80 °C. Group of vials from the same tow should be put periodically under liquid nitrogen. Do not wait until the end of sorting of all larvae from the same tow;

8) At the laboratory, vials containing larvae should be kept at −80 ºC.

2.3   Sizing, Freeze Drying, and Weighing of Larvae

1) Vials containing larvae and sea water are allowed to defrost. Once thawed, the content is emptied out gently on a small glass dish;

2) Larvae are taken from the glass dish and they are measured with an image analysis system (see Section 1) or with a stereomicroscope with an ocular ruler;

3) Then each larva is placed into a vial, with the lid being perforated with a needle. Instead of a lid, perforated parafilm can be used to seal the vial;

4) The vials are sorted in a rack and are placed in a freezer for minimum 15 minutes before being placed in the pre-cooled freeze dryer. The samples are freeze-dried for 24 hours;

5) Then place the vials containing the freeze-dried larvae in a desiccator and weigh the larvae in a microbalance, with a precision of ± 1 µg. Larvae are carefully taken from the vials with fine forceps. Larvae must be manipulated gently in order to avoid the breakage of the freeze-dried larvae; preferably touch them at the tail end;

6) After weighing, put each larva in a labelled vial and place it in a container with ice. The larva is ready for nucleic acids extraction.

If a joint study of larval condition and otolith microstructure is to be carried out on the same larvae, after weighing the larvae, otoliths should be carefully removed from the larvae. In order to do this, freeze-dried larvae are rehydrated with a drop of Tris buffer pH 8 (see Section 2.4.1, Homogenization reagents). The rehydration facilitates the otoliths extraction. Once otoliths have

been extracted, the larva is put back into its vial. The larva is now ready for the nucleic acids extraction (for otolith extraction procedures, see Section 1).

2.4 Extraction of Nucleic Acids

The reagents and the methodology recommended for homogenization and determination of nucleic acids from fish larvae are described below. In order to avoid contamination of samples by workers, especially contamination with RNase, disposable gloves should be worn during the preparation of reagents and during the manipulation of samples.

2.4.1 Homogenization reagents

Tris buffer, pH 8.0

Tris 0.05 M: Tris (hydroxymethyl-aminomethane) 6.057 g l^{-1}
NaCl 0.1 M: Sodium chloride 5.844 g l^{-1}
EDTA 0.01 M: Ethylene diamine tetraacetic acid disodium salt dihydrate 3.722 g l^{-1}
Adjust the buffer to pH 8 with concentrated HCl
Store at 4 ℃

Sodium dodecyl sulphate (SDS)

Working solution: 0.7 %

2.4.2 Homogenization and centrifugation

Larvae are homogenized in the extraction buffer (Tris buffer pH 8) containing SDS. The volume of homogenization buffer will depend on the larval size or larval weight:

Larva weight < 300 µg	200 µl buffer
Larva weight 300–2000 µg	500–700 µl buffer
Larva weight > 2000 µg	2000 µl buffer

The SDS (a detergent) is included to improve the extraction of nucleic acids. The effect of this detergent during the homogenization step, at different concentrations, was tested during the IV intercalibration exercise. The results of this analysis have shown that there were significant differences (ANOVA $p<0.05$) in the nucleic acids estimated between samples that were treated with SDS and those that were not treated with SDS. Homogenates treated with SDS yielded higher RNA and DNA content than homogenates that were not treated. The samples were treated with two different concentrations of SDS (0.01 % and 0.2 % final concentrations). Samples treated with 0.2 % SDS yielded more DNA than samples treated with 0.01 % SDS and similar values of RNA were estimated with both concentrations of SDS. However, there were analytical problems when 0.2 % SDS was used. The SDS at 0.2 % precipitated at low temperatures, whereas there was no precipitation when 0.01 % SDS was used. It was also observed that large aggregates of organic matter tended to follow the crystallized 0.2 % SDS into the supernatant. Therefore, SDS at a final concentration of 0.01 % was included as an essential reagent for homogenization of larvae.

During the intercalibration (Exercise V) carried out within the PARS project, two methods of homogenization were tested: sonication with an ultrasonic probe and use of a shaking mill. The results from the different homogenization procedures showed no differences in the yield of nucleic acids when the shaking mill or sonication were used. In consequence, both methods can be recommended for the homogenization of fish larvae.

Sonication

When using the ultrasonic processor, the probe must never be allowed to come in contact with anything but the buffer. Insert the probe deep enough below the surface of the buffer to inhibit aerosoling or foaming. Foaming substantially reduces cavitation (powerful shearing action). In ultrasonic processing, the molecular motion in the buffer causes temperature elevation, especially with small volumes. This can be prevented by immersing the vial in an ice bath. The larvae are homogenized by means of two ultrasonic pulses of short duration (5–10 s). Set the output control and the scale taking into account that decreasing the power increases the processing time. Processing at a lower power setting without foam is more efficient than processing at a higher power setting with foam. After the homogenization of each larva, the probe of the ultrasonic processor must be rinsed with distilled water and dried with a piece of paper.

Shaking mill

The vial containing the larva with buffer is filled with different sized glassbeads (diameter 2.0 mm and 0.2 mm), which should fill the tip of the vial. The glassbeads are rapidly moved in the solution and lead to the disruption of the larval tissue. A shaking time of 15 minutes on a mill Retsch Type MM-2 is enough to complete the homogenization of the tissue.

Homogenization protocol

Taking into account the above recommendations, the following protocol for larvae homogenization can be used:

1) Put the larva in a vial and add 690 µl of Tris buffer plus 10 µl of SDS (0.7 %). The final concentration of SDS in the sample is 0.01 %. Place the vial in an ice bath.

2) The larva is homogenized by means of two ultrasonic pulses of short duration (5–10 s).

3) In order to improve the extraction of nucleic acids, once the larvae have been homogenized, the samples are shaken in a vortex for 15 minutes, at 0 ℃.

4) The vials containing the homogenates are centrifuged for 8 minutes at 6,000 rpm (approximately $3,800 \times \mathbf{g}$) at –2 ℃.

2.4.3 Homogenate treatment

Homogenates must be processed immediately in order to avoid loss of nucleic acids. An internal exercise (Intercalibration Exercise IV) was carried out to test the influence of the time of storage on the RNA and DNA content. The results of this analysis showed there were significant differences in the DNA and RNA content after 24 hours' storage in the refrigerator ($p<0.05$), suggesting that samples should be measured immediately after processing.

Extraction and purification method

There are several protocols that are used for the extraction and purification of nucleic acids. A common treatment is to wash the homogenate with a mixture (1:1) of phenol and chloroform/isoamyl alcohol (24:1). The aim of this treatment is to remove proteins and lipids from the nucleic acids solutions. The homogenates are extracted several times with this mixture, and finally they are treated only with chloroform/isoamyl alcohol (24:1) to remove phenol traces. During the intercalibration exercises the effect of extraction with phenol-chloroform/isoamyl alcohol on the RNA and DNA content was tested. The results of the

Intercalibration Exercise IV revealed that the effect of the purification washes led to a decrease in the amount of DNA and RNA content and lower recovery rates compared with the method which does not use purification wash steps.

Moreover, there are some problems associated with the purification washes procedure. The phenol should be buffered at pH 8 with Tris in order to avoid loss of nucleic acids (Maniatis *et al.,* 1989). Also, the phenol should be redistilled at 160 ºC to remove oxidation products, such as quinones, that cause crosslinking of RNA and DNA. Likewise, aggregations of RNA sometimes occur following phenol extraction (Slater, 1984).

Therefore, the method recommended for nucleic acids determination in fish larvae does not include purification wash steps.

2.5 Fluorimetric Determination of Nucleic Acids

Fluorimetric methods are the most sensitive to determine small concentrations of nucleic acids. All fluorimetric methods used to determine nucleic acids are based on the use of fluorescent dyes that react specifically with nucleic acids. During the intercalibration exercises carried out in the PARS project two different approaches, using two fluorescent dyes: ethidium bromide and bisbenzimide, were tested to determine DNA and RNA content. Ethidium bromide is an intercalating reagent that reacts specifically with based-paired regions of DNA and RNA; therefore, it is used for total nucleic acids determinations (DNA+RNA). Bisbenzimide is a fluorescent dye that reacts specifically with DNA, due to the preference of bisbenzimide for adenide-thymine base areas.

There are no dyes specific for RNA, so the fluorescence due to RNA must be calculated by subtracting the fluorescence of DNA from total fluorescence due to DNA+RNA.

The first approach uses ethidium bromide to determine total nucleic acids in an aliquot of the homogenate. In this approach, the DNA concentration is determined independently by treating an aliquot of the homogenate with RNase, followed by incubation at 37 ºC for 30 minutes. The RNA is destroyed during this enzymatic treatment, giving only the DNA. The DNA concentration is then determined using ethidium bromide.

In the second approach, both fluorescent dyes are used. The fluorescence measured with ethidium bromide corresponds to total nucleic acids (DNA+RNA), whereas the DNA concentration is determined by bisbenzimide.

Based on the results of the intercalibration exercises it was decided to use the first approach, which involves the use of ethidium bromide followed by a treatment with RNase. This method is relatively fast, and involves a smaller number of hazardous chemicals than the other methodologies. Moreover, several authors have reported problems when bisbenzimide is used. Precipitation of DNA can occur if concentrated solutions are mixed (Cooney and Mathew, 1984). High self-fluorescence of the homogenate and the influence of quenching substances disturb the DNA determination with this dye (Clemmesen, 1993).

2.5.1 Reagents

RNase

Type I-AS: from bovine pancreas
Stock solution: 1 mg ml^{-1}. Preserve the stock solution at –80 ºC
Working solution: 0.2 mg ml^{-1}.

Ethidium bromide (EB)

Working solution: 0.1 mg ml^{-1} if measurements are made in cuvettes
Working solution: 0.01 mg ml^{-1} if measurements are made in a continuous fluorimeter
Cover the flask with aluminium foil and store in a refrigerator at 4 ℃. Take aliquots of this solution for analysis.

Note on safety: Ethidium bromide is a powerful mutagen and is also toxic. Use suitable gloves and protection when working with solutions of this reagent. Use a mask and be especially careful when weighing out the dry powder; preferably buy it already dissolved. After use, the solutions of this reagent should be decontaminated by one of the methods described in Maniatis *et al.* (1989).

DNA

Type I: from calf thymus
Stock solution stored at 4 ℃.

2.5.2 Treatment with RNase

The centrifuged homogenates are split into two aliquots. If proteins are also going to be determined, the homogenate must be split into three aliquots: two for nucleic acids determination and one for protein analysis.

RNase digestion protocol

1) One aliquot of 100 µl of supernatant is transferred to a vial for RNA+DNA determination.

2) The other aliquot of 100 µl is transferred to another vial for DNA measurement.

3) Add 10 µl of RNase to the sample for DNA analysis. Shake and place the sample in a warm plate at 37 ℃ for 30 minutes. A thermostatic water bath can be used if a warm plate is not available. The vials are placed in a piece of polyspan, which has holes where the vials are placed.

4) In order to have the same volume in both vials, 10 µl of Tris buffer is added to the sample for RNA+DNA determination, and kept on ice.

2.5.3 Fluorescence measurements

If the fluorescence measurements of DNA and RNA are made in cuvettes, we recommend the use of disposable methacrylate cuvettes. These cuvettes do not need to be washed, whereas quartz or glass cuvettes have to be washed and dried after each measurement. Therefore, use of methacrylate cuvettes involves less manipulation of hazardous chemicals than quartz or glass cuvettes, making the measurements faster.

During the intercalibration exercises, different excitation and emission wavelengths were tested. The RNA and DNA concentrations were significantly different when measured at different wavelengths. The RNA and DNA concentrations were higher at λexc 360 nm–λem 590 nm than at λexc 324 nm–λem 594 nm. Therefore, λexc 360 nm–λem 590 nm are the wavelengths recommended for fluorescence measurements of nucleic acids.

If measurements will be carried out in cuvettes, two cuvettes must be prepared for each larva: one cuvette for DNA determination and the other for DNA+RNA determination.

The procedure is as follows:

1) Switch on the fluorimeter and allow it to warm up for 30 minutes.

2) Add 1,800–1,850 µl of Tris buffer to the cuvettes, depending on the larval size. If very small larvae have to be analysed for nucleic acid content, the total volume in the cuvette can be reduced to 600 µl by using half-micro cuvettes.

3) Depending on the larval size, take between 50–100 µl of the sample treated with RNase for DNA determination. Take the same volume for DNA+RNA determination and add it to the cuvette containing Tris buffer.

4) Ethidium bromide has a self-fluorescence and this must be taken into account. At least three blanks must be prepared in the same way the samples have been prepared, but without larvae.

5) Set the excitation wavelength at λexc 360 nm and the emission wavelength at λem 590 nm. Set the excitation and emission slits to obtain the maximum sensitivity of the fluorimeter. During the measurement, use a suitable integration time (10 s) in order to obtain a high signal-to-noise ratio.

6) Read the fluorescence of the blanks. The fluorescence of the blank will be the average fluorescence of the three blanks. The fluorescence of the blanks must be subtracted from the fluorescence of the samples.

7) After the sample has been added to the cuvette, add 100 µl of ethidium bromide, and gently mix the content of the cuvette with the tip of the micropipette, taking care that the solution does not come into contact with the pipette.

8) Allow a reaction time of 2 minutes. Afterwards, read the fluorescence of the sample using a excitation wavelength of λexc 360 nm and an emission wavelength of λem 590 nm. The fluorescence remains constant for over one hour.

Fluorescence intensity is affected by temperature, so measurements should always be done at the same temperature.

Calibration curves

There are two ways to obtain the concentrations of DNA and RNA in cuvettes:

- Concentrations can be obtained from comparisons with calibration curves made with DNA and RNA standards, relating concentrations with fluorescence intensity:

    DNA $(\mu g \, l^{-1}) = a \times$ Fluorescence
    RNA $(\mu g \, l^{-1}) = b \times$ Fluorescence

Where a and b are the slopes of the calibration curves.

- The concentration of RNA can be also obtained from a calibration curve made with DNA standard and using a conversion factor of 2.2 (Le Pecq and Paoletti, 1966), through the following equations:

    RNA $(\mu g \, l^{-1}) = ($RNA fluorescence $\times 2.2)/a$ (1)

Where:

- RNA fluorescence = (DNA+RNA) fluorescence – DNA fluorescence   (2)

    DNA fluorescence is the fluorescence of the sample treated with RNase
    (DNA+RNA) fluorescence is the fluorescence of the untreated sample

- *a* is the slope of the DNA calibration curve.

Care must be taken with the selection of DNA standard. Different standards can lead to different calibration curves, and therefore can have a great influence on the DNA and RNA content and on the estimation of the RNA/DNA ratio. During the third intercalibration exercise with larval homogenates, the DNA content in different samples was much more similar between laboratories than in previous calibration exercises when a common DNA standard in the different laboratories was used.

Similar variances were obtained for the RNA/DNA ratios (intercalibration IV) when calculated from the RNA and DNA calibration curves and when calculated from the RNA to DNA fluorescence relationship given by Le Pecq and Paoletti (1966). However, interlaboratory differences in RNA content and RNA/DNA ratio were lower when the conversion factor of Le Pecq and Paoletti (1966) was used. Therefore, we recommend calculating the RNA concentration using equation (1).

Preparation of DNA and RNA standards

The DNA used as a standard is DNA Type I (from calf thymus), which can be acquired from several commercial companies.

1) Weigh around 25 mg of DNA and dissolve in 100 ml of Tris buffer pH = 8 (containing SDS 0.01 %).

2) The DNA takes several days to dissolve. In order to speed up this process, a soft agitation using a magnetic stirrer should be applied for several hours. During this process the solution must be kept on ice.

RNA Type III (from bakers' yeast) can be used if a standard of RNA will be prepared.

1) Weigh around 25 mg of RNA and dissolve in 100 ml of Tris buffer pH = 8 (containing SDS 0.01 %). Prepare the solution as above described for the DNA standard.

2) The solution must be kept at 4 ºC.

The DNA and RNA concentrations in the standards are determined on a spectrophotometer at 260 nm against a blank of Tris buffer. The DNA concentration is calculated taking into account that one OD unit (Absorbance) at 260 nm corresponds to 50 µg ml^{-1} DNA (double stranded). The RNA concentration is calculated taking into account that one OD unit (Absorbance) at 260 nm corresponds to 40 µg ml^{-1} RNA.

1 OD (260 nm) = 50 µg ml^{-1} DNA (double stranded)
1 OD (260 nm) = 40 µg ml^{-1} RNA

The standard curves are prepared with the standard solutions described above. When absorbance measurements are made in the UV region, quartz cuvettes must be used. Care must be taken to ensure that the curve remains linear over the range of nucleic acid concentrations tested. Since

the measurement conditions can vary between days due to instrument noise and temperature, a standard curve should be generated on every assay day.

2.5.4 Protocol for RNA and DNA determination in fish larvae

The following protocols summarize the items discussed in this section on the methodology used to determine nucleic acids in fish larvae.

1) Thaw the larva and measure its standard length.

2) Put the larva in a vial with the lid having been perforated with a needle. Freeze-dry for 24 hours.

3) Place the freeze-dried larva in a desiccator and weigh the larva in a microbalance.

4) Put the larva in a vial, add 690 µl of Tris buffer pH 8 plus 10 µl of SDS 0.7 %. Homogenize the larva with two pulses (5–10 s) of ultrasound.

5) Shake the samples in a vortex mixer for 15 minutes, at 0 ℃.

6) Centrifuge the homogenate at 6,000 rpm for 8 minutes, at −2 ℃.

7) Take two aliquots of 100 µl of the supernatant. Transfer one aliquot to a vial for DNA measurement and the other aliquot to the vial for RNA+DNA determination.

8) Add 10 µl of RNase (0.2 mg ml^{-1}) to the vial for DNA determination, and 10 µl of Tris buffer to the vial for RNA+DNA determination.

9) Incubate the vial for DNA analysis in a warm plate at 37 ℃ for 30 minutes. Keep the vial for RNA+DNA analysis on ice.

10) Take 100 µl of each vial to measure fluorescence.

11) For a total volume of 2 ml, add 1800 µl of Tris buffer pH 8 plus 100 µl of sample plus 100 µl of ethidium bromide to the cuvette.

12) Measure the fluorescence of the samples against a blank: Excitation at 360 nm, emission at 590 nm.

If the fluorescence measurements are made on a continuous flow spectrofluorimeter, the following should be considered: After RNase digestion, transfer with a dispenser 0.7 ml of EB at room temperature to the vials for DNA and DNA+RNA determination, allowing a reaction time of 2 minutes. Measure the fluorescence at the wavelength indicated until stable values are obtained. Always start analysing RNA+DNA samples.

A flow cell is used with the following parameters:

- sample 10

- delay 10

- integrate 10

- purge 20 (distillate water)

Calculation of the DNA and RNA concentrations per larva

The concentrations of nucleic acids per larva are calculated as follows:

1) Subtract the fluorescence of the blank from the fluorescence of the samples.

2) Calculate the concentration of DNA (μg ml^{-1}) in the cuvette using the standard curve obtained for DNA.

3) Obtain the fluorescence due to RNA by subtracting the DNA fluorescence from the (DNA+RNA) fluorescence (equation 2).

4) Calculate the concentration (μg ml^{-1}) of RNA in the cuvette using the Le Pecq and Paoletti equation (equation 1).

5) To obtain μg of nucleic acids in the cuvette, multiply the concentration by the total volume of the solution in the cuvette.

6) The result is divided by the volume of homogenate added to the cuvette. This result is then multiplied by the total volume used during the digestion with RNase (volume of homogenate+volume of RNase).

7) Divide the last result by the volume of homogenate used for RNase digestion (volume of homogenate). Finally, multiply this result by the total volume of buffer (Tris+SDS) used during the homogenization step.

These operations can be summarized in the following equations, if the volumes are those shown in the protocol recommended above:

DNA/larva (μg)= (((((DNA fluorescence/a μg ml^{-1}) $\times$ 2 ml)/100 μl) $\times$ 110 μl)/100 μl) $\times$ 700 μl

RNA/larva (μg)=(((((RNA fluorescence $\times$ 2.2/a μg ml^{-1}) $\times$ 2 ml)/100 μl) $\times$ 110 μl)/100 μl) $\times$ 700 μl

Where:

- a (μg ml^{-1}) is the slope of the DNA calibration curve;

- RNA fluorescence = (DNA+RNA) fluorescence − DNA fluorescence;

- 2.2 is the Le Pecq and Paoletti conversion factor.

2.6 Precision of the Method

The coefficient of variation (CV) of the proposed method for nucleic acids determination in fish larvae is relatively low when larval homogenates are analysed. During intercalibration exercises carried out within the PARS project, the results of IV intercalibration exercises with sardine larvae homogenates showed that the CVs for RNA were higher than those for DNA. The CVs for RNA were in the range of 3.1–4.1 %, whereas for DNA they were between 0.6–4.3 %. The CVs for RNA/DNA ratios were even higher than the CVs for RNA, 3.9–7.7 %. This higher variability in RNA/DNA ratio is due to the fact that RNA fluorescence is always calculated by subtracting the fluorescence of DNA from the total fluorescence due to DNA+RNA, so any error in quantification of DNA or total nucleic acids is multiplied, enhancing the variability in RNA/DNA ratios (Hovenkamp and Witte, 1991; Suthers *et al.*, 1996). In order to obtain a high precision of the measurements, great care should be taken at all steps of the analytical method.

When intercalibration exercises were carried out with individual larvae (herring and sardine), the CV was higher than the CV obtained for homogenates. The CVs for RNA/DNA ratio of sardine larvae ranged from 11.63–19.89 % for smaller larvae (10–12 mm), while for the larger larvae (18–20 mm) the CVs were between 12.03–14.03 %. The increase of the variability is probably due to the intrinsic variability of each larva.

2.7 Joint Study of Otolith Microstructure and Nucleic Acids

The joint study of otolith microstructure and nucleic acids in the same larva can be a very useful tool to study long-term growth rates and recent growth in fish larvae, and their relation with environmental variables. In Section 2.3 the procedure for otolith extraction in joint studies is explained (for otolith extraction procedures, see Section 1). However, great care must be taken when joint studies are made, because otolith extraction can lead to a loss of larval tissue and therefore a loss in nucleic acid content. The effect of otolith extraction on nucleic acid content was tested during the PARS project. Sardine larvae analysed during the VI intercalibration (without otolith extraction) were compared with sardine larvae from the same sampling. The latter were analysed jointly for otolith microstructure and larval condition. Only larvae within the same size classes were compared. The results of ANCOVA on Ln-transformed data using the Ln of larval size as covariate showed that otolith extraction caused a significant decline (ANCOVA $p < 0.0001$) of the RNA and DNA content, and also a significant decline in the RNA/DNA ratio.

2.8 References

Bergeron, J.P. 1997. Nucleic acids in ichthyoplankton ecology: a review, with emphasis on recent advances for new perspectives. Journal of Fish Biology, 51: 284–302.

Bergeron, J.P., Boulhic, M., and Galois, R. 1991. Effet de la privation de nourriture sur la teneur en ADN de la larve de sole (*Solea solea* L.). ICES Journal of Marine Science, 48: 127–134.

Buckley, L.J. 1982. Effects of temperature on growth and biochemical composition of larval winter flounder (*Pseudopleuronectes americanus*). Marine Ecology Progress Series, 8: 181–186.

Buckley, L.J. 1984. RNA-DNA ratio: an index of larval fish growth in the sea. Marine Biology, 80: 291–298.

Buckley, L., Caldarone, E.M., and Ong, T.-L. 2000. RNA-DNA ratio and other nucleic acid-based indicators for growth and condition of marine fishes. Hydrobiologia, 401: 265–277.

Bulow, F.J. 1987. RNA-DNA ratios indicators of growth in fish: A review. *In* The age and growth of fish, pp. 45–64. Ed. by R.C. Summerfelt and G.E. Hall. Iowa State University Press, Ames, Iowa, USA.

Chícharo, M.A., Chícharo, L., Valdés, L., López-Jamar, E., and Ré, P. 1998. Estimation of starvation and diel variation of the RNA/DNA ratios in field-caught *Sardina pilchardus* larvae off the north of Spain. Marine Ecology Progress Series, 164: 273–283.

Clemmesen, C. 1993. Improvement in the fluorimetric determination of the RNA and DNA content of individual marine fish larvae. Marine Ecology Progress Series, 100: 177–183.

Clemmesen, C. 1994. The effect of food availability, age or size on the RNA/DNA ratio of individually measured herring larvae: laboratory calibration. Marine Biology, 118: 377–382.

Clemmesen, C., and Doan, T. 1996. Does the otolith structure reflect the nutritional condition of a fish larva? - Comparison of otolith structure and biochemical index (RNA/DNA ratio) determined on cod larvae. Marine Ecology Progress Series, 138: 33–39.

Cooney, C.A., and Mathew, H.R. 1984. The isolation of satellite DNA by density gradient centrifugation. *In* Molecular Methods in Biology, Vol 2. Nucleic Acids. Ed. by J.M. Walker. Humana Press, Clifton, New Jersey, USA.

Ferron, A., and Leggett, W.C. 1994. An appraisal of condition for marine fish larvae. Advances in Marine Biology, 30: 217–303.

Folkvord, A., and Hunter, J.R. 1986. Size specific vulnerability of northern anchovy (*Engraulis mordax*) larvae to predation by fishes. Fishery Bulletin U.S., 84: 859–869.

Foster, A.R., Houlihan, D.F., Hall, S.J., and Burren, L.J. 1992. The effects of temperature acclimation on protein synthesis rates and nucleic acid content of juvenile cod (*Gadus morhua* L.). Canadian Journal of Zoology, 70: 2095–2102.

García, A., Cortés, D., and Ramírez, T. 1998. Daily larval growth and RNA and DNA content of the NW Mediterranean anchovy *Engraulis encrasicolus* and their relations to the environment. Marine Ecology Progress Series, 166: 237–245.

Houde, E.D. 1987. Fish early life dynamics and recruitment variability. American Fisheries Society Symposium, 2: 17–29.

Hovenkamp, F. 1990. Growth differences in larval plaice *Pleuronectes platessa* in the Southern Bight of the North Sea as indicated by otolith increments and RNA/DNA ratios. Marine Ecology Progress Series, 58: 205–215.

Hovenkamp, F., and Witte, J.I.J. 1991. Growth, otolith growth and RNA/DNA ratios of larval plaice *Pleuronectes platessa* in the North Sea 1987 to 1989. Marine Ecology Progress Series, 70: 105–116.

Le Pecq, J.B., and Paoletti, C. 1966. A new fluorometric method for RNA and DNA determination. Analytical Biochemistry, 17: 100–107.

Maniatis, T., Fritsch, E.F., and Sambrook, J. 1989. Molecular cloning: A laboratory manual. Cold Spring Harbor Laboratory Press, New York.

Purcell, J.E., Siferd, T.D., and Marliave, J.B. 1987. Vulnerability of larval herring (*Clupea harengus pallasi*) to capture by the jellyfish *Aequorea victoria*. Marine Biology, 94: 157–162.

Ramirez, T., Cortes, D., and Garcia, A. 2001. Growth of North Alboran Sea sardine larvae estimated by otolith microstructure, nucleic acids and protein content. Journal of Fish Biology, 59: 403–415.

Rice, J.A., Miller, T.J., Rose, K., Crowder, L.B., Marschall, E.A., Trebitz, A.S., and DeAngelis, D.L. 1993. Growth rates variation and larval survival: inference from an individual-based size-dependent predation model. Canadian Journal of Fisheries and Aquatic Sciences, 50: 133–142.

Richard, P., Bergeron, J.P., Boulhic, M., Galois, R., and Person-Le Ruyet, J. 1991. Effect of starvation on RNA, DNA and protein content of laboratory reared larvae and juveniles of *Solea solea*. Marine Ecology Progress Series, 72: 69–77.

Rooker, J.R., and Holt, G.J. 1996. Application of RNA:DNA ratios to evaluate the condition and growth of larval and juvenile red drum (*Sciaenops ocellatus*). Marine and Freshwater Research, 47: 283–290.

Slater, R.J. 1984. The extraction of total RNA by the detergent and phenol method. *In* Molecular Methods in Biology, Vol 2. Nucleic Acids. Ed. by J.M. Walker. Humana Press, Clifton, New Jersey, USA.

Suthers, I.M., Cleary, J.J., Battaglene, S.C., and Evants, R. 1996. Relative RNA content as a measure of condition in larval and juvenile fish. Marine and Freshwater Research, 47: 301–307.

Westerman, M., and Holt, G.J. 1994. RNA:DNA ratio during the critical period and early larval growth of the red drum *Sciaenops ocellatus*. Marine Biology, 121: 1–9.

Wright, D.A., and Martin, F.D. 1985. The effect of starvation on RNA:DNA ratios and growth of larval striped bass, *Morone saxatilis*. Journal of Fish Biology, 27: 479–485.

3   OTOLITH MICROCHEMISTRY

3.1   Introduction

Otolith microchemistry (OMC) is the study of the minor and trace elemental composition of otoliths. It is a relatively recent and rapidly developing discipline that has a wide range of potential uses. These applications can be categorized into two broad areas: those that are concerned with the reconstruction of a fish's environmental history and those that are concerned with the separation of fish populations. Otolith microchemistry has been used to reconstruct temperature history of fishes (Radtke, 1989; Radtke *et al*., 1990; Townsend *et al*., 1989, 1992, 1995), to detect anadromy (Kalish, 1990), to assess migratory events (Radtke *et al*., 1996; Tzeng and Tsai, 1994), as well as being used to differentiate among fish stocks (Campana, 1994; Campana *et al*., 1994; Edmonds *et al*., 1991, 1992; Gunn *et al*., 1992; Kalish *et al*., 1996; Milton *et al*., 1997; Proctor *et al*. 1995; Severin *et al*., 1995; Thresher *et al*., 1994; Thorrold *et al*., 1998). A recent review by Campana (1999) provides a comprehensive overview of the range of applications for which otolith microchemistry has been used. The success of otolith microchemistry is reliant upon two key properties of otoliths. Firstly, the otolith is metabolically inert; thus, when otolith material is deposited it is neither resorbed nor reworked. Secondly, the otolith grows throughout the lifetime of a fish. Trace element uptake is believed to reflect the elemental composition of the environment, which, in combination with these properties, suggests that the potential exists for the otolith to act as a complete age-structured record of exposure history to the environment (Campana *et al*., 1997).

A wide range of techniques (mostly developed from geological applications) has been used to analyse otolith composition and, hence, access the information that they may contain. These techniques can broadly be categorized into those that are able to sample small, discreet regions of the otolith and bulk techniques that are used to analyse the composition of whole otoliths. The analytical capabilities of, and sample requirements for, each method vary a great deal. Therefore, the technique used in a particular study is highly dependent upon the type of question being addressed.

Isotopic ratios in otoliths can also be analysed to reveal valuable information about fish life history. No isotope ratios were measured in the course of the PARS project, to enable the participants to concentrate on a thorough evaluation of a smaller range of techniques.

The following section is a practical guide to the three techniques that are most commonly used for otolith microchemistry work: WDS (Wavelength dispersive spectrometry), SB-ICPMS (Solution based-inductively coupled plasma mass spectrometry), and LA-ICPMS (Laser ablation-inductively coupled plasma mass spectrometry). The usefulness of each technique is assessed for stock separation and environmental history studies on larval herring (*Clupea harengus*) and sardine (*Sardina pilchardus*). Two partners within the PARS project conducted experiments:

Partner 3: University of Liverpool, Port Erin Marine Laboratory, Isle of Man;

Partner 6: LAASA, IFREMER, Brest, France

3.2 Specimen Collection

Experiments were conducted on wild and laboratory-reared specimens during the course of the PARS project.

Wild herring larvae were provided from annual larval surveys conducted in the North Sea by the Netherlands Institute for Fisheries Research, the Institute for Marine Fisheries Kiel, Germany, and the Danish Institute for Fisheries Research. Sampling methods for herring larvae are described in Section 2.2, above. Larvae were sorted and identified onboard and preserved in 70 % alcohol; they were held in airtight plastic containers before being sent to the laboratory for analysis.

3.2.1 Laboratory-reared larvae

Two sets of experiments were conducted on laboratory-reared herring larvae in order to test hypotheses relating to: a) the effects of trace metal concentrations in rearing waters upon otolith composition; and b) the effects of rearing temperature upon otolith composition. Herring reared in the mesocosm experiments were also analysed.

3.2.2 Sardine larvae/juveniles

Sardine larvae and juveniles were obtained from annual surveys of larval abundance conducted in the Mediterranean Sea and from the Atlantic Ocean bordering the northwest coast of the Iberian Peninsula.

3.3 Sizing and Storage of Larvae

As prolonged storage in alcohol has been shown, in some studies, to affect the elemental composition of the otolith (Proctor and Thresher, 1998; de Pontual, unpublished data), it is recommended that larvae are stored in alcohol for as short a time as possible before analysis. The effects of alternative storage methods, such as freezing in liquid nitrogen, have not yet been investigated. Prior to otolith removal, the standard length of each specimen was recorded. Where possible (herring only), the stage of larval development was noted using the Doyle (1977) staging index.

3.3.1 Larval length measurements

Larvae were gently removed from the alcohol using non-metallic forceps (to avoid possible metal contamination) and placed in a small quantity of 70 % alcohol in a watch glass or plastic petri dish. Contorted larvae were straightened as much as possible whilst taking great care to avoid damage. A digital image of each larva was obtained using a compound microscope to which was fitted a digital video camera and frame grabber. Digital images were stored and the standard lengths of larvae were measured from the image using calibrated Optimas image analysis software.

3.4 Otolith Extraction and Storage

Otoliths of all specimens were dissected out under a binocular dissecting microscope. Metal dissecting implements are likely to cause contamination of the otolith and should not be used for otolith removal. A range of alternative implements is available for "clean" dissection work. Fine glass needles were made by heating thin glass capillary tubing with a naked flame (Bunsen) and pulling gently apart. This method can create glass needles of varying thicknesses which are ideal for the removal of very small otoliths, but which are fragile. Larger otoliths are easily removed using short lengths of fibre optic filament mounted with thermo-plastic resin in a glass capillary tube. Commercially available, fine-tipped, ceramic forceps may be suitable for the removal of the largest otoliths. All dissecting implements should be thoroughly cleaned in an ultrasonic bath prior to use. Whenever possible, dissections should be carried out in a laminar flow hood. All otolith extractions were carried out on ultrasonically cleaned standard glass slides.

Better visualization of the very smallest otoliths can be achieved by altering the lighting conditions. The use of transmitted light and polarizing filters may improve the visibility of the otolith during dissection. Once removed from the head, any adhering tissue should be teased away from the otolith using the fine dissecting implements. Otoliths should be rinsed in small drops of distilled water before storage. Sterile plastic cell culture plates provide an ideal numbered storage system for very small otoliths. Transfer of the otolith to these plates can be difficult and is best performed whilst the otolith is still damp. After sealing with Parafilm, the plates can be stored until the otoliths are required. If necessary, digital images of the whole otolith can be taken to facilitate orientation at a later stage. Subsequent treatment of the otolith is dependent upon the type of technique used.

3.5 Wavelength Dispersive Spectrometry (WDS) Analysis

3.5.1 Introduction

Several electron probe microanalysis techniques are theoretically capable of being used to study otolith composition. These probes, including WDS, all function in a similar manner. A finely focused high-energy electron beam generates characteristic X-rays in the parts of the specimen exposed to it. Chemical variation on a micrometer scale across a specimen's surface can be measured in this way. An electron probe microanalysis system generally consists of two parts:

1) the electron optics "column", which provides the specimen-handling and imaging facilities coupled to the vacuum system;

2) the X-ray spectrometer or "analyser", which unravels each X-ray spectrum into individual element peaks; the associated computer software translates peak intensities into element concentrations or element distribution patterns, by comparison with analytical standards (certified reference materials (CRMs)).

WDS analyses can be used to make analyses with a very fine spatial resolution (< 5 µm), but relatively poor limits of detection (LODs) mean that it is only suitable for the measurement of the more abundant elements (> 200 ppm). In otolith studies, this restricts WDS studies to the measurement of Ca, Na, Sr, K and, less reliably, Mg. For quantitative analyses, the specimen must have an exceptionally flat and highly polished surface if reliable data are to be generated. A concise introduction to electron beam methods, including WDS, is given by Gill (1997). The following section describes larval otolith preparation for analysis by WDS.

3.5.2 Sample preparation

Preparation techniques were developed to facilitate the WDS microprobe analysis of large numbers of very small larval otoliths, in many cases < 50 µm diameter. A large number (up to 30) of similarly sized otoliths were mounted in close proximity to one another in the central region of a glass slide using Spurr low-viscosity resin. The slides were etched with a cross-hatch pattern of lines, using a glass etching pen, to prevent delamination of the resin during subsequent grinding and polishing. Care was taken to ensure that the orientation of each otolith was the same. The slides were then placed at the bottom of shallow silicone rubber moulds (6 mm deep). A very thin layer of Spurr resin was poured into the mould, covering the slide evenly to a height just above the otolith. After polymerization in a 70 $^{\circ}$C oven for approximately 9 hours, the slides were gently removed from the moulds. Preparations were then ground by hand, initially using P1200 wet carborundum paper on a Buehler Metaserv polishing wheel. A slow and cautious approach should be adopted in order to prevent the over-grinding and polishing of the otolith. Regular checks of the plane of polishing should be made throughout preparation to prevent such an occurrence. When the desired plane was reached (usually the plane of the primordium), specimens were polished to the extremely smooth, glass-like surface required for WDS analysis using progressively 6 µm, 1 µm, and 0.25 µm water-based, diamond polishing suspensions. Excellent polishing results can also be obtained using diamond-based aerosols. Otolith surface quality was monitored during preparation using a compound microscope equipped with an epi-illumination attachment. After the final polish, preparations were cleaned in an ultrasonic bath containing distilled water and stored in a desiccator. It is useful to obtain digital images of the whole otolith preparation at this point as an aid to orientation for analysis. Larger otoliths, for example the otoliths of juvenile sardine, can be prepared individually. Individual otoliths were embedded in a suitable resin (Spurr or Aradilte) using conventional petrological moulds. Grinding and polishing methods were the same as for the slide-mounted specimens. Non-conducting materials such as otoliths are required to be vacuum coated with a thin conducting film to prevent charge build-up on the specimen surface. Specimens are typically carbon coated to a thickness of 20 nm. Preparations should then be earthed to the specimen holder using a colloidal graphite paint such as Aquadag. All analyses were carried out using a Cameca Cambax SX-50 WDS (located at IFREMER, Brest, France).

3.5.3 WDS operation

3.5.3.1 Standards

Quantitative analysis demands the use of standards of accurately known composition. It is normally sufficient to use one standard per element being analysed. The standards typically used for the analysis of the elemental composition of otoliths are given in Table 1. In WDS analysis it is usual to measure all of the relevant standards once or twice during every analysis session.

Table 1. Operating conditions and standards used for WDS analyses of larval herring and sardine otoliths.

Voltage	15 Kv		
Current	10 nA		
Beam size (diameter)	5 µm		
Element/Crystal	**Collecting time (s)**	**Standards**	
Na (TAP)	140	Albite	$NaAlSi_3O_8$
Ca (PET)	40	Wollastonite	$Ca_2Si_2O_6$
K (PET)	140	Orthoclase	$KAlSi_3O_8$
Sr (TAP)	120	Strontium sulphate	$SrSO_4$
Mg (TAP)	120	Forsterite	$MgSiO_4$

3.5.3.2 Operating conditions

The operating conditions of the WDS used in the analysis of otolith composition in the PARS study are given in Table 1. These conditions are very similar to those used in other studies of otolith composition (see Toole and Nielsen, 1992). It should be noted that changes in operating conditions could have a marked effect upon the resolution, precision, and accuracy of WDS measurements. These effects are dependent upon the element under analysis. A detailed account of the effects of changes in operating conditions upon data quality in otolith studies is provided in Gunn *et al*. (1992). Generally, increases in beam power (increased accelerating voltage or current or reduction in beam diameter) will give better resolution and accuracy for most elements but will eventually lead to specimen damage and erroneous results. Operating conditions should always be reported and kept constant between analyses.

3.5.3.3 Sample analysis

Measurements of the concentrations of calcium (Ca), strontium (Sr), sodium (Na), magnesium (Mg), and potassium (K) can be made at discrete spots on the specimen surface. These may be along pre-determined radials extending from the core to the edge of each otolith or single points within a growth band or region. Radial analyses are usually required when changes in otolith composition during the life of an individual are being assessed. However, for stock analysis studies it is likely that elemental composition data are required from a particular region of the otolith, usually the core or outermost edge (representing the most recently deposited material). Most WDS machines are fully automated. After selecting the analysis points and entering their respective coordinates, the WDS is able to run unattended. Background counts were measured with each analysis and corrected X-ray intensity ratios of elements were calculated using the ZAF method (see Gill, 1997). The lower limits of detection (LOD) for each element analysed are usually calculated as $3 \times$ St.Dev. of the background (blank) elemental concentration. Typical beam diameters of between 1 µm and 5 µm enable analyses of very small temporal changes in elemental composition of an otolith to be made. However, it should be noted that changes in micro-increment width within an otolith could affect the data quality. Small impressions created by the probe during analysis can usually be seen on the specimen surface when subsequently viewed under epi-illumination (Figure 5). These impressions are very useful for assigning back-calculated ages for each analysis point.

3.5.3.4 WDS data

Output from the WDS is in the form of the per cent weight for each element analysed. This is usually converted to the more widely cited value of PPM (parts per million) or is expressed as a ratio of the elemental concentration to the Ca concentration. The relatively low sensitivity of the

WDS means that, in many analyses, values for the less abundant elements fall below the limits of detection. There is no consensus within the literature about how best to deal with these observations. In the PARS study, elemental concentrations below the lower limit of detection were excluded from all further analysis.

3.6 Inductively Coupled Plasma Mass Spectrometry (ICPMS)

3.6.1 Introduction

ICPMS (Inductively Coupled Plasma Mass Spectrometry) is a recently developed multi-elemental technique in which positive ions generated from a specimen by means of an inductively coupled plasma (high temperature electrically conductive ionized gas) are extracted into a low-resolution mass analyser. This is designed to analyse a wide mass spectrum very rapidly providing near-simultaneous determination of most elements in the periodic table at levels down to 10 pg ml^{-1}. It provides a rapid and versatile method for trace element analysis of materials (which may be in the form of solutions, slurries or solids) over a wide concentration range.

Figure 5. Photomicrograph of a sagittal otolith from a Clyde herring larva reared at elevated temperature. Burn impressions caused by WDS analysis are clearly visible along the radial (arrow). Microincrements are clearly visible and are of variable width, being narrower towards the core. Consequently, the temporal resolution of each WDS analysis varies along the length of the radial.
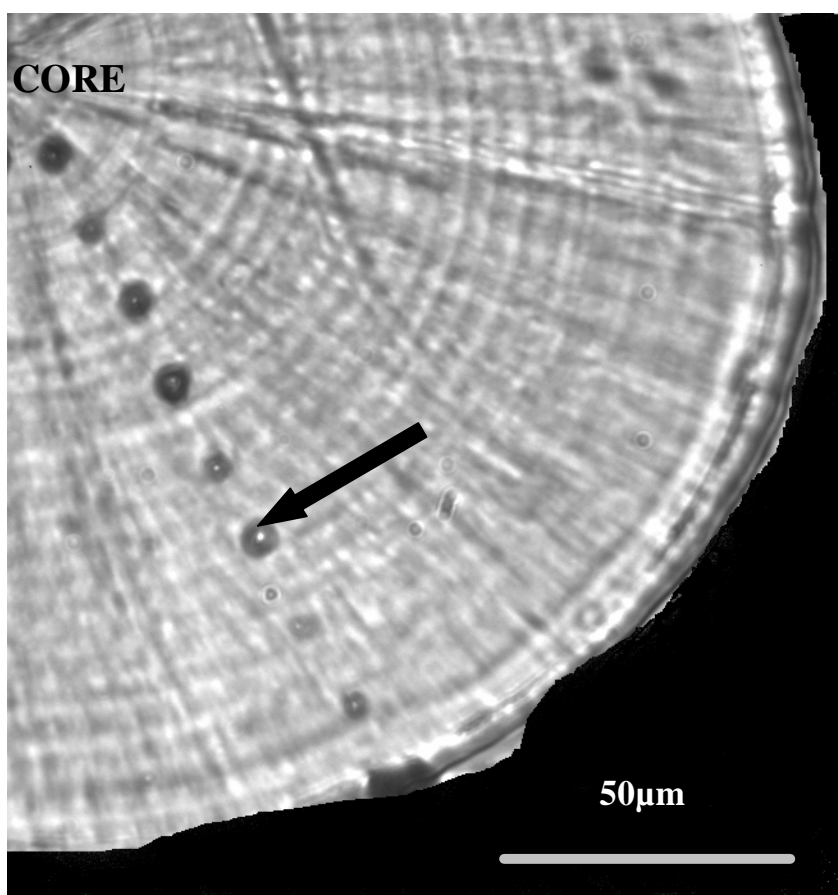
3.6.2 Solution-based ICPMS

In SB-ICPMS (Solution-based ICPMS) samples are introduced into the analyser in the form of a solution. This is converted by a nebulizer into an aerosol before it is swept into a spray chamber where large droplets of the analyte aerosol settle out into the plasma torch. On entering the high temperature region of the ICP, the aerosol is rapidly volatized, dissociated, and ionized. Charged molecules are sucked under high vacuum into the mass spectrometer, typically of the quadropole type, through two conical nickel apertures, the sampling cone and the skimmer. Non-charged particles are deflected away. Counts of analyte concentration are recorded. SB-ICPMS has very good resolution, with limits of detection in the order of <1 ng ml^{-1} for most elements under investigation. It offers rapid, accurate, and precise measurement of almost all of the elements of interest in otolith studies. The major drawback with the technique is that analyses require that the specimen must be dissolved, leading to the loss of temporal information within the otolith. At present, it is not possible to remove small amounts of material for analysis from discreet locations in the otolith using a drill. However, it is hoped that future developments will enable suitably large samples of otolith material to be obtained from discrete areas of the larval otolith for ICPMS analysis. Consequently, SB-ICPMS is more suitable for stock separation work where total trace element content within the otolith is of interest. It is not yet possible to create "life history transects" using SB-ICPMS. Full details of ICPMS operation are given in Jarvis (1997).

3.6.2.1 Otolith sample preparation

Otolith samples are prepared in much the same manner as geological samples, with the main aim of producing a homogeneous solution containing the analyte elements. After removal from the fish, otoliths are rinsed twice in acid-washed plastic micro-tubes. Samples should then be air-dried for 48 hours within a laminar flow hood to avoid contamination. Otoliths are thereafter weighed to the nearest microgram in acid-washed Teflon weighing boats. Otoliths are then dissolved in a 10 % Suprapur nitric acid solution (Merck ref. 100441) containing 10 ppb of Indium (Indium is used as an internal standard for signal control). Dilution of the solution is calculated based on otolith weight in order to obtain a dilution factor of approximately 500.

3.6.3 SB-ICPMS operation

3.6.3.1 Standards

The most commonly used calibration method for ICPMS work is the use of a set of external calibration standards (usually three plus a blank). For solution analysis, these may have a simple aqueous or acid matrix containing the analytes of interest. Several standard solutions are prepared which cover the range of expected concentrations and a calibration line is fitted to the measured data using least-squares regression analysis. Short-term fluctuations in signal may occur over a time scale of a few minutes and a gradual loss of sensitivity is not uncommon over several hours. Two approaches, drift correction and internal standardization, can be used to correct for changes in sensitivity.

3.6.3.2 Otolith analyses

For the PARS analyses, elemental concentrations in otoliths were determined using a Plasma Quad 3 (VG) ICP mass spectrometer equipped with a micro-nebulizer (Cetac MNC 100) to allow the assay of small solution volumes. 100 μl aliquots were assayed (10 s manual uptake, 3 replicates, Gilson pump adjusted to 50 μl min^{-1} flux).

3.7 Laser Ablation ICPMS

LA-ICPMS (Laser ablation ICPMS) has been developed to enable the analysis of the trace element composition of solid samples. This enables the rapid preparation of samples without the need for dilution. A laser is used to remove small amounts of material from the surface of a solid sample and the resultant vapour or vapour/particulate mixture is transported to the ICP for analysis in an argon gas flow.

3.7.1 Otolith sample preparation for LA-ICPMS

The preparation time of samples for analysis by LA-ICPMS is much less than the time required for sample preparation for WDS or SB-ICPMS. Following their removal (see Section 3.4, above) and cleaning, otoliths are embedded in a suitable low-viscosity epoxy resin such as Araldite. To speed up the analyses, a number of similarly sized otoliths can be mounted together in the same resin block. Otoliths are secured to the base of a mould using epoxy resin before the mould is filled to a depth of approximately 5 mm. After polymerization, the resin blocks (rounds) are ground to the plane of interest using a fine carborundum paper (P1200). Grinding can be performed manually or with the aid of an automatic grinder/polisher in the manner described for WDS preparation (Section 3.5.2, above). Resin blocks should be polished to a reasonably flat surface to remove obvious cracks or fissures; however, the glass-like sample surface that is necessary for WDS analysis is not required for LA-ICPMS. A final polish with a 6-µm water-based polishing suspension is usually sufficient. Unlike WDS analysis, there are no restrictions on specimen height. Following the final polish, specimens should be cleaned in an ultrasonic bath with distilled water.

3.7.2 LA-ICPMS operation

3.7.2.1 Standards

The standards that are routinely used for instrumental calibration in LA-ICPMS analyses have been developed for geochemical applications. For carbonate analysis, pressed $CaCO_3$ pellets which have been spiked with a range of trace metals at known concentration are used alongside certified reference materials (CRMs) in the form of naturally occurring carbonates. Artificially produced CRMs (e.g., NIST glass standards) are also used. Unfortunately, there is presently no CRM available for otolith applications. Otoliths can contain as much as 10 % protein and are considered as being exceptionally pure (low levels of trace metals) compared to non-biogenic carbonates. Pressed powders do not accurately reproduce the otolith matrix and otoliths may behave differently to standards during laser ablation. Semi-quantitative analysis (Pearce *et al.*, 1992a, 1992b) in which the concentration of Ca within the otolith is used as an internal standard is often used. Standards and background (argon blank) counts are taken regularly during an analysis session.

3.7.2.2 Sample analysis

Full instrumental descriptions for the LA-ICPMS are given in Pearce *et al.* (1992a, 1992b). LA-ICPMS can operate in either scanning mode, providing composition data for the whole mass spectrum, or in peak jumping mode, in which the concentrations of selected elements only are measured. Peak jumping cuts down on analytical time and improves instrumental precision and accuracy. The operating conditions of the LA-ICPMS used to analyse otoliths during the PARS project are given in Table 2. Resin rounds containing the otoliths are secured in a Perspex ablation chamber. Specimens are observed using either a binocular microscope or digital camera located above the ablation chamber. Using stepper motors, the specimen can be orientated until the region of interest is located. Cross hairs on the microscope indicate the position at which laser ablation will be carried out. Many new laser ablation systems can be pre-programmed,

allowing sampling to be carried out at pre-determined locations. The entire instrument is computer-controlled. The laser is fired on the specimen, typically creating ablation craters that are 20–30 µm in diameter (Figure 6) Ablated material is swept into the plasma by a stream of argon gas and is rapidly ionized before entering the mass spectrometer. Analyte counts are reported for each ablation (analysis). Lower limits of detection should be calculated from either gas blanks or standards during each session.

Table 2. Operating conditions and standards used in LA-ICPMS analyses of herring and sardine larval otoliths.

LA-ICPMS operating conditions	
Analysis type	Single spot
Laser mode	Q switched
Laser type	Nd:Yag (VG Laser Lab.)
Voltage (V)	700–800
Scan time (s)	30
Standards	NIST glass (610 and 612)
Analysis method	Peak jumping

Figure 6. Otolith of juvenile sardine after LA-ICPMS analysis of the core (primordium region). A large ablation crater (arrow) is shown. Scale bar = 100 µm.
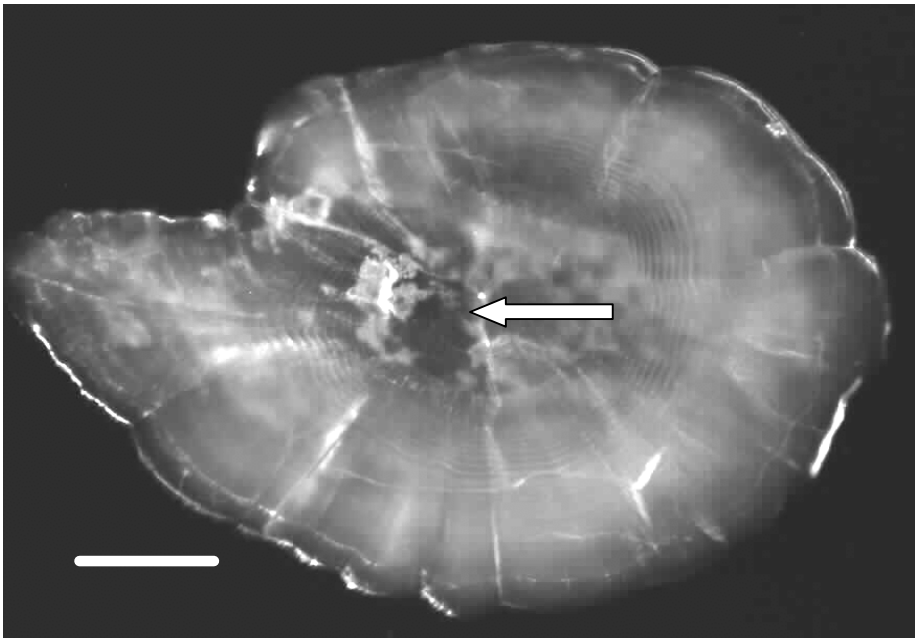
3.7.3 Disadvantages of LA-ICPMS analysis of larval otoliths

In addition to the problems associated with the unavailability of suitable reference materials for biogenic carbonate analysis, there are a few other drawbacks with the analysis of larval fish otoliths using LA-ICPMS. Although the development of laser technology is rapid, most currently available instruments produce relatively large ablation craters (20–100 µm). The resolution of the temporal signal contained within an otolith is not as good as can be achieved

using beam methods such as WDS. Repeatedly subjecting areas of the otolith to laser ablation can lead to severe cracking and eventual destruction of the sample. This is a particular problem for small larval otoliths (<100 µm radius), for which multiple sampling is often not possible.

3.8   Data Quality

Numerous issues about the quality of microchemistry data arose during the course of the PARS project. These issues were raised during the examination of the advantages and disadvantages of the techniques for otolith microchemistry. Three important issues are:

1)   the development of criteria for accepting or rejecting individual analytical results (either the results of individual points or the results of particular elements at individual points);

2)   the determination of the limits of detection (LOD) for LA-ICPMS; and

3)   the procedure for statistical treatment of data points that are below the LOD.

These issues directly impact the interpretation of microchemistry data, since they control the value (elemental concentration) assigned to each individual fish or used to represent an area of the otolith of an individual fish.

3.8.1   Data handling and analysis

There are two aspects of otolith microchemistry data that require special attention in data handling and analysis. The first aspect is that otolith composition is not homogeneous. There is variability both around the otolith and through the otolith (i.e., along a single temporal axis of the otolith, and around an arc bisecting that axis within the same temporal zone). The second aspect is that the microchemistry results often contain a number of analyses below the limits of detection, and there are no standard procedures for handling these data. During the course of the PARS project, the problems and implications inherent in each of these were examined. Sources of error in the measurement of otolith microchemistry were defined and procedures were proposed for recognizing and treating the data subject to these errors.

3.8.2   Criteria for accepting analytical results

Aberrant analytical points should be rejected from further analysis, especially when they result from irregularities and fractures in the otolith surface, the inclusion of resin in a spot, etc. In WDS analysis, spots on fractures or scratches are sometimes included if the visualization attachment has low magnification or if they occur along pre-set radial tracks. Aberrant points in WDS can easily be identified by their Ca values, and the results for that entire point should be rejected. In LA-ICPMS, fractures and resin may be included within ablation points because the exact ablation volume varies. These points should be identified by the values of abundant elements such as Sr or Mg. Continued improvements in laser technology may reduce the errors in ablation points.

The identification and rejection of aberrant points should be based on some criteria depending on how far the results deviate from the average. Local areas of contamination may also produce aberrant points which should be rejected in the same way.

3.8.3   Determining the LOD

For the WDS technique, limits of detection are estimated from the standard deviation of the background signal.

For solution-based ICPMS, limits of detection are estimated from the standard deviation of replicated measurements on blanks corresponding to the solution that is used to dissolve the samples. The estimations correspond to those of solution samples and have to be corrected by the dilution factor to refer to the otolith sample, i.e., to give the LOD in terms of ppm.

There is, up to now, no agreement regarding methods of estimation of limits of detection for LA-ICPMS, and several methods have been reported:

1) estimation from measurements on the argon gas (i.e., without ablation);

2) estimation from measurements on glass standards (e.g., NIST 612 and NIST 610);

3) estimation from measurements on powdered standards.

Each method leads to a different estimate of LODs and none of them is satisfactory owing to the lack of matching with the otolith matrix. The first one is the least "conservative" since it does not take into account the quantity of ablated material, which significantly influences the LOD values.

3.8.4 Procedure for statistical treatment of data points that are below the LOD

The existing microchemistry literature unfortunately contains few details about either the calculation of LODs or the treatment of values which fall below the LOD. It is necessary to report how the LOD is calculated, how the analytical results falling below the LOD are treated statistically, and what percentage of the results for each element fall below the LOD. This should be reported separately for each group of samples. Differences in these values between years can obscure the real comparisons between groups and produce misleading trends in data.

There are several methods for dealing with analytical results that are below the LOD, and the results for each sampling spot are usually treated element-by-element, rather than judging the entire spot:

1) include all data, using the values which are below the LOD in calculation of the mean to represent an individual or otolith area;

2) set values below the LOD to 0, using 0 in calculation of the mean to represent an individual or otolith area;

3) set values below the LOD to the LOD, using the LOD value in calculation of the mean to represent an individual or otolith area;

4) enter values below the LOD as missing values;

5) convert all values of elements which have measurements below or near the detection limit to presence/absence data and use appropriate statistical methods.

In some applications it is possible to ignore elements that have many values below the LOD. This is useful for improving the data quality when the results are subjected to multivariate analyses, as in stock separation problems.

In all cases, the occurrence of values below the LOD must be reported as:

1) per cent of all points below the LOD;

2) per cent of individuals with points below the LOD;

3) or both.

Statistical methods which include the analysis of the proportion of points below the LOD should be developed to help characterize populations and individual responses.

3.9 Conclusions

The choice of an appropriate analytical technique for otolith microchemistry (OMC) work is dependent upon what type of problem is being addressed. Table 3 is a summary of the advantages and disadvantages of the three OMC techniques discussed in this manual. It is apparent that the poor LODs restrict the use of WDS to the measurement of the concentrations of the most abundant elements within an otolith. It is generally not possible to measure the concentration of elements below 20 ppm, which rules out the majority of trace elements present in otoliths. Unfortunately, it is these elements (which tend not to be under physiological regulation) which have been shown to be of most use for stock separation studies.

Trace element analysis can, therefore, be conducted by either SB-ICPMS or LA-ICPMS. SB-ICPMS has very good LODs (sensitivity), good precision and accuracy, and is reasonably fast. However, the need to dissolve the specimen for analysis rules out this method for life history analyses. LA-ICPMS has reasonable LODs and can be used to measure trace element composition at discrete points across larger otoliths. Small, fragile larval otoliths are susceptible to damage by the laser, which may limit the technique suitability for fine resolution work. The current lack of a suitable otolith CRM for LA-ICPMS work is another constraint upon the use of this technique.

Table 3. Summary of characteristics of OMC techniques.

Characteristic	WDS	SB-ICPMS	LA-ICPMS
1. Spatial resolution	Good	None	Fair
2. Sensitivity to low concentrations	Poor	Good	Fair
3. Ease of sample preparation	Poor	Fair	Good
4. Precision of determination	Good	Good	Poor
5. Suitability for radial analyses	Good	None	Fair (large otoliths only)
6. Suitability for trace metal analysis	Poor/none	Good	Fair

3.10 References

Campana, S.E. 1994. International symposium on fish otolith research and application, January 1993, Hilton Head Island, South Carolina, USA. Reviews in Fish Biology and Fisheries, 4: 23–27.

Campana, S.E. 1999. Chemistry and composition of fish otoliths; pathways, mechanisms and applications. Marine Ecology Progress Series, 188: 263–297.

Campana, S.E., Fowler, A.J., and Jones, C.M. 1994. Otolith elemental fingerprinting for stock identification of Atlantic cod (*Gadus morhua*) using laser-ablation ICPMS. Canadian Journal of Fisheries and Aquatic Sciences, 51: 1942–1950.

Campana, S.E., Thorrold, S.R., Jones, C.M., Guenther, D., Tubrett, M., Longerich, H., Jackson, S., Halden, N.M., Kalish, J.M., Piccoli, P., De Pontual, H., Troadec, H., Panfili, J., Secor, D.H., *et al.* 1997. Comparison of accuracy, precision, and sensitivity in elemental assays of fish otoliths using the electron microprobe, proton-induced X-ray emission, and laser ablation inductively coupled plasma mass spectrometry. Canadian Journal of Fisheries and Aquatic Sciences, 54(9): 2068–2079.

Doyle, M.J. 1977. A morphological staging system for the larval development of the herring (*Clupea harengus* L.). Journal of the Marine Biological Association of the United Kingdom, 57: 859–867.

Edmonds, J.S., Caputi, N., and Morita, M. 1991. Stock discrimination by trace-element analysis of otoliths of orange roughy (*Hoplostethus atlanticus*), a deep-water marine teleost. Australian Journal of Marine and Freshwater Research, 42: 383–389.

Edmonds, J.S., Lenanton, R.C.J., Caputi, N., and Morita, M. 1992. Trace elements in the otoliths of yellow-eye mullet (*Aldrichetta forsteri*) as an aid to stock identification. Fisheries Research, 13: 39–51.

Gill, R. 1997. Modern analytical geochemistry. Longman Geochemistry Series. Longman, London.

Gunn, J.S., Harrowfield, I.R., Proctor, C.H., and Thresher, R.E. 1992. Electron probe microanalysis of fish otoliths - evaluation of techniques for studying age and stock discrimination. Journal of Experimental Marine Biology and Ecology, 158: 1–36.

Jarvis, K.E. 1997. Inductively coupled plasma mass spectrometry. *In* Modern analytical geochemistry. Ed. by R. Gill. Longman, London.

Kalish, J.M. 1990. Use of otolith microchemistry to distinguish the progeny of sympatric anadromous and non-anadromous salmonids. Fishery Bulletin, 88: 657–666.

Kalish, J.M., Livingston, M.E., and Schofield, K.A. 1996. Trace elements in the otoliths of New Zealand blue grenadier (*Macruronu novaezelandiae*) as an aid to stock discrimination. Marine and Freshwater Research, 47: 537–542.

Milton, D.A., Chenery, S.R., Farmer, M.J., and Blaber, S.J.M. 1997. Identifying the spawning estuaries of the tropical shad, terubok, *Tenualosa toli*, using otolith microchemistry. Marine Ecology Progress Series, 153: 283–291.

Pearce, N.J.G., Perkins, W.T., and Fuge, R. 1992a. Developments in the analysis of carbonates by laser ablation inductively coupled plasma mass spectrometry. Analytical Proceedings, 29: 280–281.

Pearce, N.J.G., Perkins, W.T., and Fuge, R. 1992b. Developments in the quantitative and semiquantitative determination of trace elements in carbonates by laser ablation inductively coupled plasma mass spectrometry. Journal of Analytical Atomic Spectrometry, 7: 595–598.

Proctor, C.H., and Thresher, R.E. 1998. Effects of specimen handling and otolith preparation on concentration of elements in fish otoliths. Marine Biology, 131: 681–694.

Proctor, C.H., Thresher, R.E., Gunn, J.S., Mills, D.J., Harrowfield, I.R., and Sie, S.H. 1995. Stock structure of the southern bluefin tuna *Thunnus Maccoyii* - an investigation based on probe microanalysis of otolith composition. Marine Biology, 122: 511–526.

Radtke, R.L. 1989. Strontium-calcium concentration ratios in fish otoliths as environmental indicators. Comparative Biochemistry and Physiology, 92A: 189–193.

Radtke, R., Svenning, M., Malone, D., Klementsen, A., Ruzicka, J., and Fey, D. 1996. Migrations in an extreme northern population of arctic charr *Salvelinus alpinus*: insights from otolith microchemistry. Marine Ecology Progress Series, 136: 13–23.

Radtke, R.L., Townsend, D.W., Folsom, S.D., and Morrison, M.A. 1990. Strontium - calcium concentration ratios in otoliths of herring larvae as indicators of environmental histories. Environmental Biology of Fishes, 27: 51–61.

Severin, K.P., Carroll, J., and Norcross, B.L. 1995. Electron-microprobe analysis of juvenile walleye pollock, *Theragra chalcogramma*, otoliths from Alaska: a pilot stock separation study. Environmental Biology of Fishes, 43: 269–283.

Thorrold, S.R., Jones, C.M., Swart, P.K., and Targett, T.E. 1998. Accurate classification of juvenile weakfish *Cynoscion regalis* to estuarine nursery areas based on chemical signatures in otoliths. Marine Ecology Progress Series, 173: 253–265.

Thresher, R.E., Proctor, C.H., Gunn, J.S., and Harrowfield, I.R. 1994. An evaluation of electron probe microanalysis of otoliths for stock delineation and identification of nursery areas in a southern temperate groundfish, *Nemadactylus macropterus* (Cheilodactylidae). Fishery Bulletin, 92: 817–840.

Toole, C.L., and Nielsen, R.L. 1992. Effects of microprobe precision on hypotheses related to otolith Sr/Ca ratios. Fishery Bulletin, 90: 421–427.

Townsend, D.W., Radtke, R.L., Corwin, S., and Libby, D.A. 1992. Strontium:calcium ratios in juvenile Atlantic herring *Clupea harengus* L. otoliths as a function of water temperature. Journal of Experimental Marine Biology and Ecology, 160: 131–140.

Townsend, D.W., Radtke, R.L., Morrison, M.A., and Folsom, S.D. 1989. Recruitment implications of larval herring overwintering distributions in the Gulf of Maine, inferred using a new otolith technique. Marine Ecology Progress Series, 55: 1–13.

Townsend, D.W., Radtke, R.L., Malone, D.P., and Wallinga, J.P. 1995. Use of otolith strontium-calcium ratios for hindcasting larval cod *Gadus morhua* distributions relative to water masses on Georges Bank. Marine Ecology Progress Series, 119: 37–44.

Tzeng, W.N., and Tsai, Y.C. 1994. Changes in otolith microchemistry of the Japanese eel, *Anguilla japonica*, during its migration from the ocean to the rivers of Taiwan. Journal of Fish Biology, 45: 671–683.

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