Modification and comparison of two fluorometric techniques for determining nucleic acid contents of fish larvae

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The ribonucleic acid (RNA) content and the ratio of RNA to deoxyribonucleic acid (DNA) have proven to be reliable indices of the nutritional condition of larval fish (Buckley, 1979, 1980, 1981, 1984; Wright and Martin, 1985; Buckley and Lough, 1987; Clemmesen 1987, 1988; Robinson and Ware, 1988; Canino et al., 1991; Richard et al., 1991; Canino, 1994). Cellular RNA content is correlated with the rate of protein synthesis. DNA content, which remains relatively constant in somatic tissues, may be used as an index of cell number (Bulow, 1987). The RNA/DNA ratio, therefore, reflects the protein synthesizing capability of larval fish and has been used for estimating recent in situ protein growth (see review by Bulow, 1987; Robinson and Ware, 1988; Hovenkamp, 1990; Hovenkamp and Witte, 1991).

Initial methods for determining RNA and DNA concentrations in tissue homogenates, based upon ultraviolet light absorption (Munro and Fleck, 1966; Buckley, 1979), were limited by sample size, requiring about 800 μg dry weight of tissue for a single analysis. The requirement of pooled samples of fish larvae precluded quantitation of variability among individuals. Recent development of highly sensitive fluorometric techniques for direct measurement of nucleic acid contents of marine phytoplankton (Berdalet and Dortch, 1991; Mordy and Carlson, 1991), bacteria (Mordy and Carlson, 1991), and individual fish larvae (Clemmesen, 1988, 1993; Caldarone and Buckley, 1991; Theilacker and Shen, 1993) now provides a greater choice of methods. Several protocols are based upon the fluorescence of the dye ethidium bromide (EB), when bound to nucleic acids. Fluorescence of the nucleic acid-fluorochrome complex is measured before and after digestion of RNA by RNase (Karsten and Wollenberger, 1972, 1977; Robinson and Ware, 1988; Clemmesen, 1993), or after sequential additions of RNase and DNase (Bentle et al., 1981). Total fluorescence is then partitioned into DNA and RNA components and nucleic acid concentrations are calculated indirectly by difference after enzymatic degradation.

A two-dye fluorometric method for nucleic acid analysis of individual fish larvae (Clemmesen, 1988) utilized EB to measure total sample fluorescence and the DNA-specific dye, Hoechst dye H33258 (Hoechst), to measure DNA content directly. A recent modification of this method to a single-dye procedure yields higher DNA estimates and RNA estimates comparable to the previous two-dye method (Clemmesen, 1993). Both protocols require extraction and purification of crude homogenates before analysis. While substances that interfere with sample fluorescence may be reduced or eliminated, the washing and extraction steps of both methods restrict the number of samples that can be processed in a day.

Caldarone and Buckley (1991) developed a two-dye method for determining nucleic acid contents that was coupled with an automated flow-injection analysis (FIA) system in which EB is used to estimate total nucleic acid content and Hoechst is used to measure DNA. This method has the advantage of combining high sensitivity and sample throughput rate with a simple extraction procedure. Unfortunately, an FIA system may be too costly for many laboratories. In this paper, we present an adaptation of the FIA method for conventional static fluorometric analysis (CFA) and compare results using the two procedures.

Methods

Fish larvae and juveniles from laboratory culture and field samples from six species—Atlantic cod, *Gadus morhua*; inland silverside, *Menidia beryllina*; haddock, *Melanogrammus aeglefinus*; tautog, *Tautoga onitis*; winter flounder, *Pleuronectes americanus*; and walleye pollock, *Theragra chalcogramma*—were chosen to provide different species, ages, and nutritional histories for comparison (Table 1). Fish homogenates, except those of walleye pollock, were prepared by homogenizing between 1 and 12 previously frozen individuals with deion-

Table 1

Fish species, sample abbreviations, and sources used for methods comparison. Fish sampled at discrete ages provided one homogenate at each age. (S) = starved.

Species		Sample name	Source	Age (d)	n
Atlantic cod	*Gadus morhua*	C	lab	41–61	3
		C(S)	lab (S)	41–61	3
Inland silverside	*Menidia beryllina*	S	lab	104–108	3
		S(S)	lab (S)	104–108	3
Haddock	*Melanogrammus aeglefinus*	H	lab	11,22,44	3
Tautog	*Tautoga onitis*	T	lab	20	3
Winter flounder	*Pleuronectes americanus*	F	lab	26,41,57	3
Walleye pollock	*Theragra chalcogramma*	P	field	<60	3

ized, distilled water in an Ultra-Turrax homogenizer (Tekmar Co.) with three 15-second pulses at maximum power (Caldarone and Buckley, 1991). Previously frozen walleye pollock larvae were homogenized in prechilled glass homogenizers. Six aliquots of homogenate were pipetted into 1.5-mL polyethylene vials and immediately frozen at –80°C until analysis of three aliquots by FIA and three aliquots by CFA.

Sample extraction procedures followed those outlined by Caldarone and Buckley (1991). Homogenates were extracted in 1% sarcosine (N-lauroylsarcosine), TRIS-EDTA (5.0 mM TRIS-HCl, 0.5 mM EDTA, pH 7.5) buffer for 30 minutes at room temperature, mixed vigorously in a sample vortexer for 10–15 seconds, then extracted for another 30 minutes. Aliquots were diluted with TRIS-EDTA buffer to yield a final concentration of 0.1% sarcosine, then centrifuged at room temperature for 5 minutes at 2,500 times gravity ($\times$ g) for CFA and 12,000 $\times$ g for FIA. The supernatant was recovered for estimation of nucleic acid concentrations by FIA or CFA. Sarcosine, like other anionic detergents, fluoresces at excitation and emission wavelengths used in the analyses. To reduce this effect, samples extracted in 1% sarcosine required a 10-fold dilution with TRIS-EDTA buffer before FIA (Caldarone and Buckley, 1991). In the modified CFA procedure, a final sample concentration of 0.0125% sarcosine produced an acceptable background fluorescence (blank) value of approximately 5% of the highest nucleic acid standard.

Differences in the total sample volumes required by FIA and CFA procedures required modifications to the concentrations of nucleic acid standards, fluorochrome reagents, and sample volumes of fish homogenates. Nucleic acid standard ranges and homogenate sample volumes were chosen to encompass the typical range of concentrations encountered by each method during routine analysis of individal

fish larvae. Working standards of RNA and DNA were prepared as described by Caldarone and Buckley (1991) by serial dilution of previously frozen stock solutions with 0.1% sarcosine in TRIS-EDTA buffer. RNA standards (Sigma Chemical Co., St. Louis, MO, Type IV, calf liver) ranged from 1.97 to 17.68 μg·mL^{-1} for FIA and from 0.41 to 13.14 μg·mL^{-1} for CFA. DNA standards (Boehringer-Mannheim Corp., Indianapolis, IN, high molecular weight, calf thymus) ranged from 0.16 to 1.52 μg·mL^{-1} for FIA and from 0.07 to 2.37 μg·mL^{-1} for CFA. Estimates of contamination of the calf liver RNA standard by DNA, determined by fluoresecence in Hoechst, was less than 1% by weight. A 100-μL "spike" of homogenate from walleye pollock larvae was added to serial dilutions of RNA and DNA standards in order to estimate the recovery efficiencies using the CFA protocol.

Fluorochrome working reagents of EB (137.5 ng·mL^{-1}) and Hoechst (25 ng·mL^{-1}) prepared for FIA in TRIS-EDTA buffer according to Caldarone and Buckley (1991) were modified by reducing the sodium chloride (NaCl) concentration in the Hoechst reagent from 0.2 N to 0.1 N and the pH from 7.5 to 7.0. Working dye solutions of 2.0 μg·mL^{-1} EB and 5.0 μg·mL^{-1} Hoechst prepared for CFA at the same pH and NaCl concentration as for FIA provided a sensitive linear response to the standards while maintaining a low background fluorescence.

The RNA and DNA concentrations were estimated for each aliquot. In addition, the amount of endogenous fluorescence (sample fluorescence in the absence of fluorochrome dye) was determined for most homogenate samples (21 for FIA, 17 for CFA) by substituting an equal volume of TRIS-EDTA buffer for the fluorochrome working reagent in the assay procedure.

The FIA system for nucleic acid determination is fully described by Caldarone and Buckley (1991). A 50-μL sample is injected into a reagent stream con-

taining one of the fluorochrome dyes. The injected sample is mixed and transported with the reagent to the fluorescence detector which continuously records the fluorescence at 525 nm excitation and 600 nm emission for EB, or 356 nm excitation and 458 emission for Hoechst. The sample fluorescence is displayed as a peak, whose area is proportional to the concentration (Caldarone and Buckley, 1991). Modification of this procedure for CFA required a minimal total volume (sample plus fluorochrome reagent) of 2 mL in order to be measured accurately by the fluorometer (Shimadzu RF–540 spectrofluorophotometer, Shimadzu Corp., Kyoto, Japan), which was adapted to use 12×75 mm borosilicate glass test tubes as cuvettes. For all samples, except the homogenates of larval pollock, a 0.1-mL aliquot of extracted sample was combined with 0.9 mL of TRIS-EDTA buffer and 1.0 mL of fluorochrome working reagent (EB or Hoechst). For larval pollock homogenates, a 0.03-mL aliquot was combined with 0.97 mL of TRIS-EDTA buffer prior to addition of 1.0 mL of the fluorochrome reagents. The sample-fluorochrome mixture was incubated at room temperature for 15–30 minutes before fluorescence was measured at the same excitation and emission wavelengths as in the FIA procedure. Initial trials indicated that maximum sample fluorescence was obtained within 15 minutes and was stable for more than 4 hours at room temperature.

Calculations of nucleic acid concentrations were identical for both methods. First, endogenous sample fluorescence was subtracted from total sample EB or Hoechst dye fluorescence. Sample DNA concentrations were estimated directly from fluorescence in Hoechst dye by using a DNA-Hoechst standard curve. The computed DNA concentration was used to estimate the fluorescence contribution by DNA to the total sample EB-fluorescence by using a DNA-EB standard curve. Fluorescence due to DNA-EB was subtracted from the total sample fluorescence and the remaining fluorescence was assumed to be due to RNA. The RNA concentration was then estimated by using an RNA-EB standard curve.

The relationships between mean RNA and DNA concentration and RNA/DNA ratios of the fish homogenates predicted by FIA and CFA methods were analyzed by using a geometric mean regression procedure (Ricker, 1984) that describes the linear central trend between two independent estimates of the variate.

Results

Standard calibration curves indicated that detection limits for CFA are about 0.07 μg·mL^{-1} for RNA and

0.03 μg·mL^{-1} for DNA, similar to the values detectable with automated FIA (Caldarone and Buckley, 1991). The precision of both methods was comparable; mean coefficients of variation, V_x (standard deviation as a percentage of the mean), for triplicate determinations from each homogenate averaged 5 to 7% for RNA and 3 to 4% for DNA over a broad range of estimated sample concentrations. Recovery of DNA standards from six replicate "spikes" of larval pollock homogenate with the CFA method averaged 99.5 ± 0.9% in Hoechst and 99.2 ± 2.9% in EB, and recovery of "spiked" RNA standards averaged 94.8 ± 6.0% in EB.

Mean nucleic acid concentrations and RNA/DNA ratios of fish homogenates were generally lower when estimated by CFA relative to FIA (Fig. 1). RNA concentration was most strongly correlated between the two methods and DNA concentration less so (Table 2). The ratio of RNA to DNA was only moderately correlated between the two methods and provided the poorest basis for comparison. Intermethodological calibration between FIA and CFA results was achieved by the application of regression coefficients (Table 2) to mean nucleic acid concentrations and RNA/DNA ratios estimated by CFA (Fig. 2).

Homogenates prepared from larval stages of the gadid species (*Gadus morhua*, *Melanogrammus aeglefinus*, and *Theragra chalcogramma*), tautog, *Tautoga onitis*, and winter flounder, *Pleuronectes americanus*, exhibited negligible endogenous fluorescence, regardless of fluorochrome, over a 3- to 4-fold range of nucleic acid concentrations (Table 3). Endogenous fluorescence was highest for juvenile inland silverside and juvenile winter flounder.
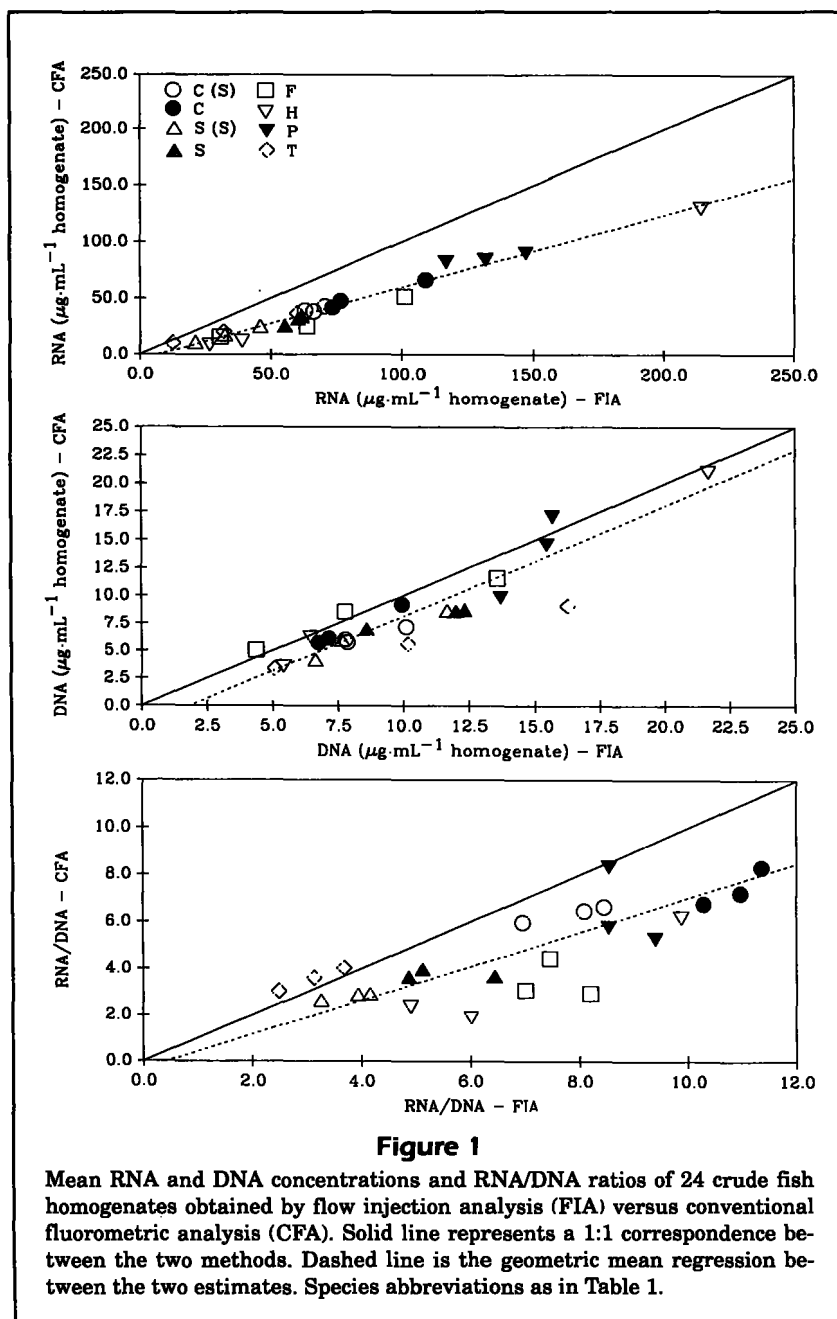
Discussion

Modification of the FIA method described by Caldarone and Buckley (1991) to conventional fluorometry produced an assay protocol with comparable

Table 2

Geometric mean functional regression coefficients describing mean RNA and DNA concentrations (μg·mL^{-1} homogenate) and RNA/DNA ratios of 24 fish homogenates determined by conventional fluourometric analysis (CFA) regressed upon estimates obtained by flow injection analysis (FIA).
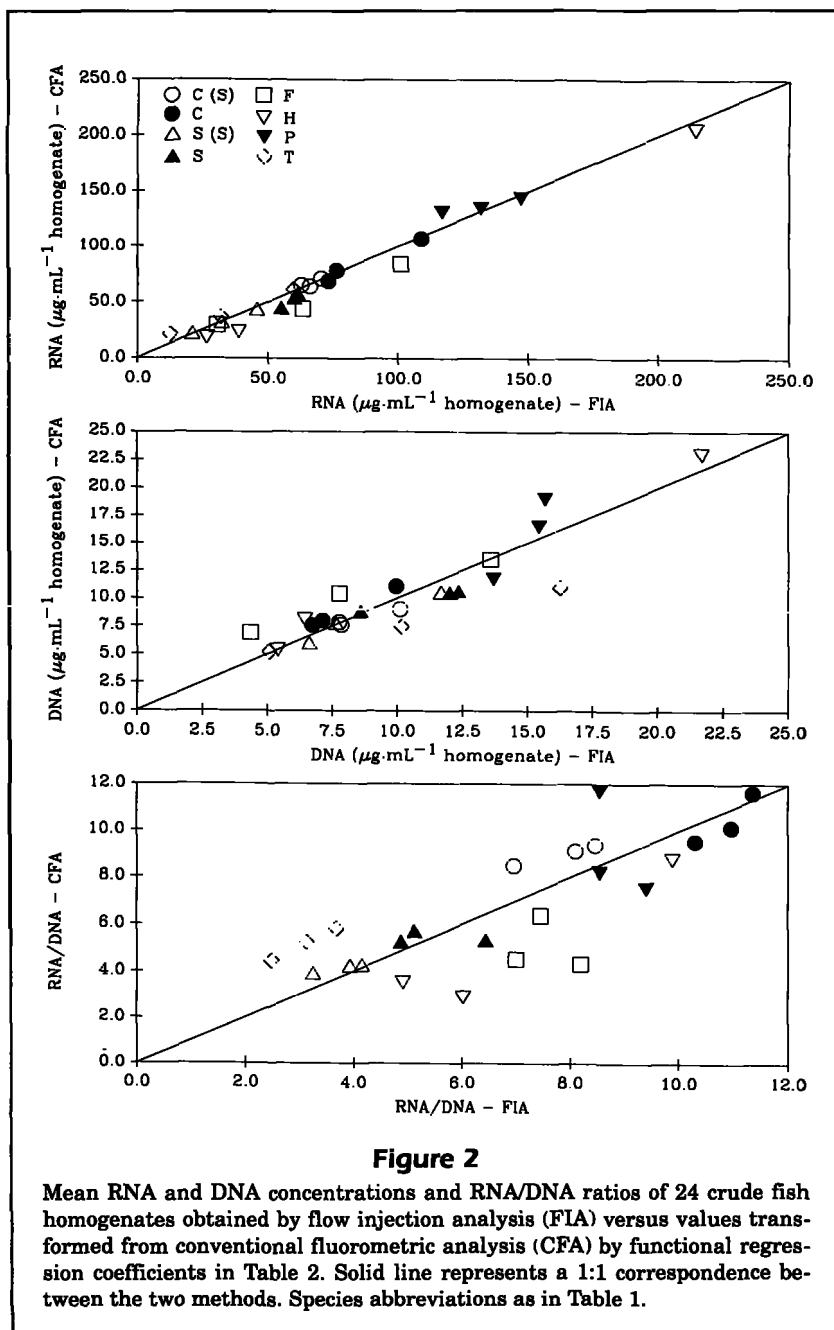
Variate	Y-intercept	Slope	r^2
RNA	–5.318	0.652	0.972
DNA	–1.795	0.991	0.808
RNA/DNA	–0.309	0.733	0.569

Figure 1

Mean RNA and DNA concentrations and RNA/DNA ratios of 24 crude fish homogenates obtained by flow injection analysis (FIA) versus conventional fluorometric analysis (CFA). Solid line represents a 1:1 correspondence between the two methods. Dashed line is the geometric mean regression between the two estimates. Species abbreviations as in Table 1.

sensitivity, precision, and sample throughput. Mean coefficients of variation (V_x) for DNA concentration estimated by FIA and CFA in this study (3 to 4%) are similar to those reported for replicate assays of a pooled fish homogenate using FIA (Caldarone and Buckley, 1991), another two-dye procedure (Clemmesen, 1988), and a single-dye method (Clemmesen, 1993). In this study, the mean V_x values for RNA concentration were 3 to 5% higher when determined by FIA and CFA for multiple homogenates than for FIA estimates of a single, pooled homogenate (Caldarone

and Buckley, 1991) but are comparable to those reported by Clemmesen (1993) for RNA estimates of pooled fish homogenate using a single-dye technique.

The RNA and DNA concentrations of fish homogenates were consistently lower when estimated by CFA compared with FIA. Calibration between the two methods by functional regression relationships established a reasonable basis for comparison of results (Fig. 2), although considerable differences in mean estimated nucleic acid concentrations and RNA/DNA were still evident. We emphasize that sample homo-

Figure 2

Mean RNA and DNA concentrations and RNA/DNA ratios of 24 crude fish homogenates obtained by flow injection analysis (FIA) versus values transformed from conventional fluorometric analysis (CFA) by functional regression coefficients in Table 2. Solid line represents a 1:1 correspondence between the two methods. Species abbreviations as in Table 1.

genates for this study were chosen from six fish species and assayed across far greater ranges of age and nucleic acid concentrations than reported previously (Clemmesen 1988, 1993; Caldarone and Buckley, 1991; McGurk and Kusser, 1992) in order to broaden the scope of comparison and statistical inference. A comparative study of FIA and CFA techniques within the more limited range of sample concentrations encountered during routine processing of individual larvae of a single species would have undoubtedly yielded higher correlations between estimates.

The FIA and CFA procedures differ primarily in the choice of instrumentation as both use sample extraction by sarcosine. Caldarone and Buckley (1991) reported that nucleic acids in larval fish samples (<200 μg dry weight) were completely extracted in one hour at room temperature. Sample nucleic acid concentrations of fish homogenates in this study were prepared to be similar to those obtained from assays of individual larvae, suggesting that incomplete or differential extraction of homogenates by sarcosine between FIA and CIA is un-

Table 3

Mean endogenous fluorescence[1] of fish homogenates as a percentage of total fluorescence in Hoechst 33258 (Hoechst) or ethidium bromide (EB) fluorochromes. Samples are from larvae unless otherwise noted. (S) = starved.

Species	Sample name	Age (d)	Hoechst		EB	
			FIA	CFA	FIA	CFA
Atlantic cod	C	41–61	<3	<2	<1	3
Atlantic cod (starved)	C(S)	41–61	<3	<2	<1	3
Inland silverside (juvenile)	S	104–108	38	33	<1	12
Inland silverside (juvenile, starved)	S(S)	104–108	40	37	<1	12
Haddock	H	11	<3	<2	<1	<2
		22	<3	<2	<1	<2
		44	<3	<2	<1	2
Tautog	T	20	<3	<2	<1	<2
Winter flounder	F	26	<3	<2	<1	<2
		41	<3	<2	<1	<2
Winter flounder (juvenile)		57	19	14	<1	6
Walleye pollock	P	<60	<3	<2	<1	2

[1] Endogenous sample fluorescence occurs in the absence of fluorochrome dye.

likely. The consistently lower estimates of nucleic acids with the CFA method, relative to the FIA method, implies a lower ratio of fluorescence yield of samples to standards. One possibility is that the effective sample concentration at the detector may be greater in CFA compared with FIA. In FIA, the time between the mixing of dye and sample is precisely controlled but the ratio of dye to sample is unknown. The concentrations of the fluorochrome working reagents used in FIA were increased (14× and 100× for EB and Hoechst, respectively) in order to saturate the standards for the CFA modification. A higher effective sample concentration at the detector (possibly making the samples less available to the dye), coupled with a longer pathlength, may explain the lower fluorescence yield of the CFA samples relative to the standards. The higher correlation between the methods for RNA estimates than for DNA was an unexpected result; RNA content is calculated indirectly (total sample fluorescence minus estimated fluorescence due to DNA) and, presumably, is more subject to measurement error than is direct fluorometric determination of DNA.

Fluorescence by compounds other than nucleic acids represents a potential source of interference in any fluorometric assay. The level of endogenous sample fluorescence should be determined by preliminary analyses when a new fish species or developmental stage is being investigated. Crude tissue homogenates exhibit fluorescence characteristics not found in commercial preparations of nucleic acids that appear to be related to tissue type and developmental stage of the fish. Caldarone and Buckley

(1991) found that winter flounder and American sand lance, *Ammodytes americanus*, larvae and the nucleic acids used as standards exhibited negligible amounts of endogenous or residual fluorescence, whereas winter flounder postlarvae and juvenile muscle and liver had levels ranging from approximately 5 to 55% of total fluorescence in EB or Hoechst dyes. Clemmesen (1993) reported endogenous fluorescence values ranging up to 40% in Hoechst determinations of the DNA content of herring, *Clupea harengus*, larvae. In this study, only homogenates of the juvenile inland silverside and juvenile winter flounder exhibited high levels of endogenous fluorescence in Hoechst dye or EB (Table 3), providing further evidence of an ontogenetic effect. Gadid larvae, of approximately the same age as the juvenile winter flounder, displayed negligible amounts of endogenous fluorescence, suggesting that systematic differences may also exist.

Interference in nucleic acid estimation from contaminants of crude homogenate preparations has been reported previously (Brunk et al., 1979; Mordy and Carlson, 1991; Clemmesen, 1993). McGurk and Kusser (1992) compared three fluorescence methods for quantitating nucleic acids in Pacific herring, *Clupea pallasi*, larvae. Of the three, the method incorporating the most extensive purification steps (Clemmesen, 1988) resulted in higher estimates of RNA content and RNA/DNA ratios than the other two methods, suggesting that higher yields may have resulted from the elimination of substances interfering with accurate fluorometric quantitation. However, quenching of nucleic acid fluorescence by contaminants in the samples does not appear to be sig-
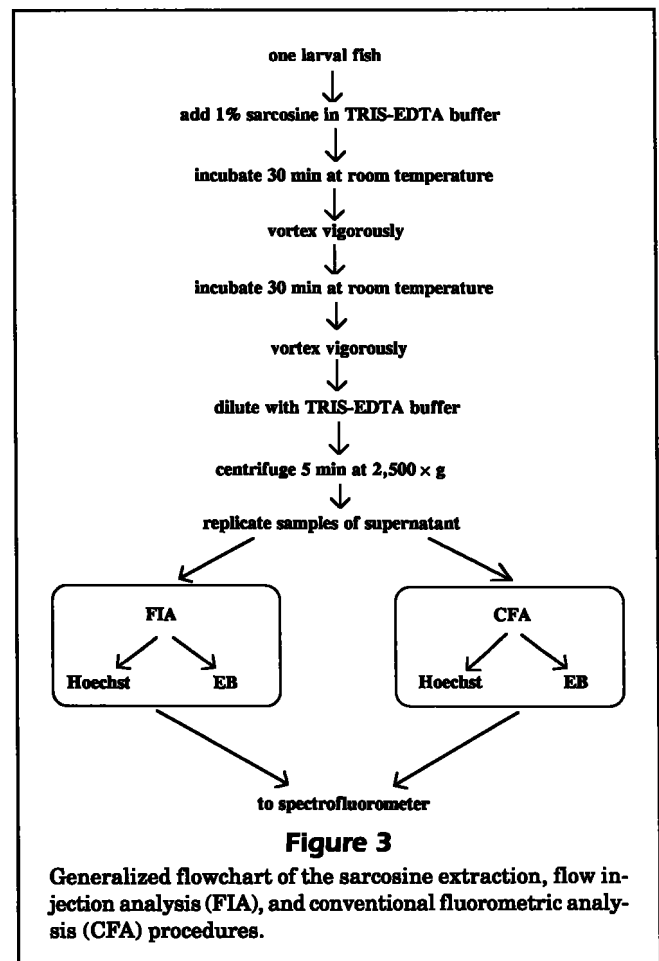
nificant in either the CFA or FIA techniques using the sarcosine extraction procedure with larval fish. Recoveries of crude homogenate "spikes" added to nucleic acid standards by FIA (Caldarone and Buckley, 1991) and CFA (this study) are similar to those reported for more purified extracts (Clemmesen, 1993). McGurk and Kusser (1992) reported higher RNA contents and RNA/DNA ratios for yolk-sac herring larvae analyzed with the Clemmesen method (1988) and suggested that fluoresecence absorbance by yolk components may be reduced by the purification steps in that assay. However, when a homogenate of winter flounder yolk-sac larvae was "spiked" with nucleic acid standards and subjected to FIA with a sarcosine extraction procedure, recoveries of calf thymus DNA standards remained unchanged and those for calf liver RNA standards only declined by 3 to 5% (Caldarone, Unpubl. data).

RNA/DNA indices have proven useful as indicators of condition in a wide variety of fish species. When coupled with data on water temperature, and calibrated with laboratory-reared larvae, estimates of recent growth in the field can be obtained (Buckley, 1984). However, this study and others illustrate a common problem with the application of fluorescent techniques to estimation of nucleic acid levels in fish and other biosamples, and the need for inter-calibration. Given the disparity in estimates of RNA and DNA contents due to the method of analysis (McGurk and Kusser, 1992; this study) and choice of nucleic acid standards (Caldarone and Buckley, 1991), no direct intermethodological comparisons of data can be made without intercalibration between analytical methods, as done by McGurk and Kusser (1992), Clemmesen (1993), Mathers et al. (1994), and this study. It is inappropriate to compare RNA/DNA ratio values with published data unless the same methods and standards are used. Also, the generalized growth equation in Buckley (1984) uses RNA/DNA ratios determined with an ultraviolet light absorption method that cannot be directly applied to ratios determined with other analytical procedures without running an intercalibration between the two methods. Alternatively, the relation between RNA/DNA, temperature, and growth must be determined for laboratory-reared larvae by using the analytical method of choice before a growth equation can be applied to fish larvae collected in the field.

A general assay protocol for FIA and CFA is presented in Figure 3. For this study, fish larvae were pooled and homogenized to provide adequate replicates for methods comparison. On a routine basis, individual larvae may be frozen in 1.5-mL microcentrifuge vials, then extracted in the same vial, re-

ducing processing time and errors associated with sample transfer. The actual volumes of 1% sarcosine and TRIS-EDTA buffer may have to be determined empirically depending upon the larval fish size and sample volume required for spectrofluorometric analysis. A trained operator can process approximately 80 samples for RNA and DNA determinations as well as standards in eight hours using CFA and in five hours with FIA. We do not routinely assay replicate sample aliquots or correct for endogenous sample fluorescence when preliminary estimates are less than 3% of total sample fluorescence.

The "best" method for nucleic acid analysis of larval fish may well be determined by sample size, instrumentation, the presence of interfering substances, or the need to compare values to previously published data. Flow-injection analysis (Caldarone and Buckley, 1991) is a sensitive, precise assay with a simple extraction procedure and high sample throughput. The modified CFA protocol presented here retains those advantages, extends them to a more inexpensive method using static fluorometry, and provides an intercalibration between the two methods.

Figure 3

Generalized flowchart of the sarcosine extraction, flow injection analysis (FIA), and conventional fluorometric analysis (CFA) procedures.

Acknowledgments

The authors thank L. Buckley, G. Theilacker, D. Busch, A. Kendall Jr., and K. Bailey for their helpful comments on earlier versions of the manuscript and three anonymous reviewers for their critical reviews. S. Picquelle provided assistance in the statistical treatment of the data. This study was conducted as part of the Fisheries Oceanography Coordinated Investigations (NMFS, Seattle) and the GLOBEC Cod Recruitment Studies (NMFS, Narragansett) and represents FOCI contribution 0195 and GLOBEC contribution 0194.

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