Intercalibration of four spectrofluorometric methods for measuring RNA-DNA ratios in larval and juvenile fish

E.M. Caldarone, C. Clemmesen, E. Berdalet, T.J. Miller, A. Folkvord, G.J. Holt, M.P. Olivar & I.M. Suthers

Background information

The ratio of tissue RNA to DNA (RNA/DNA) is a widely used index of recent growth and nutritional condition in larval and juvenile fish. To date, however, no standard technique for measuring nucleic acids in fish has been established. Because methodological details (e.g. choice of standards or fluorophore) can affect the value of RNA/DNA, researchers using different analytical protocols have been unable to compare ratios directly. Here, we report on the results of an international inter-laboratory calibration of four spectrofluorometric protocols to quantify nucleic acids.

Objective

To determine whether it is possible to standardize RNA/DNA values obtained from different analytical protocols to allow for meaningful comparisons of the data.

Material & Methods

Replicate sets of five tissue samples and two standards (16S & 23 S rRNA, lambda DNA) were supplied to each of five researchers for RNA/DNA analysis with their own spectrofluorometric protocol and standards (individual standards).

Two approaches were investigated to intercalibrate the resulting data:

1) the use of common standards (supplied standards) (Caldarone & Buckley 1991)

2) a normalization procedure based on the ratio of the slopes of the standards (as described in Berdalet et al. 2005)



Tissues Analyzed

Species	Sample abbreviation	Life Stage	Sampling location	Tissue sampled
Houting *Coregonus oxyrhynchus*	Co	Larval	Laboratory reared Kiel, Germany	Whole fish
Herring *Clupea harengus*	Ch-l	Larval	Field-caught , International Herring Larvae Survey, ICES Orkney/Shetland area	Whole fish
Herring *Clupea harengus*	Ch-j1, Ch-j2	Juvenile	Field-caught Kiel Canal, Germany (Baltic)	Whole fish minus head, gut, tail fin
Cod *Gadus morhua*	Gm	Juvenile	Mesocosm-reared Flodevigen, Norway	Muscle filet

Analytical protocols used by each laboratory

Laboratory abbreviation	Fluorophore	Fluorometer type	Excitation wavelength (nm)	Emission wavelength (nm)	Protein dissociator	Enzymes	Individual RNA standard	Individual DNA standard
EBsarco1	Ethidium bromide	Microplate	530	590	N-lauroylsarcosine	RNase	18S &28S rRNA	Calf thymus
EBsarco2	Ethidium bromide	Microplate	545	575	N-lauroylsarcosine	RNase	18S &28S rRNA	Calf thymus
EBSDS	Ethidium bromide	Microplate	355	590	Sodium dodecyl sulfate (SDS)	RNase	16S & 23S rRNA	Lambda DNA
EBproK	Ethidium bromide	Cuvette	365	590	Proteinase K	RNase followed by DNase	Baker's yeast	Calf thymus
SYBR	SYBR Green I	Cuvette	490	520	N-lauroylsarcosine	RNase and DNase independently	18S &28S rRNA	Calf thymus

Calculations for Normalization Procedure

Rationale: The slopes of the RNA and DNA standard curves have a major influence in determining the value of nucleic acids in a sample. Normalizing to the ratio of the slopes of the standard curves will allow the RNA/DNA values from different protocols to be more meaningfully compared.

1. Concentrations of nucleic acids are determined using a linear standard curve F=mx+b where:
   x = concentration of the nucleic acid ([RNA] or [DNA])
   F = fluorescence units
   m = slope
   b = intercept
2. Solving for concentration (x) yields: $[RNA] = (F_R\text{-}b)/m_{RNA}$ and $[DNA] = (F_D\text{-}b)/m_{DNA}$
3. Since the intercepts are close to zero the equations simplify to:
   $[RNA] = F_R/m_{RNA}$ $[DNA] = F_D/m_{DNA}$
4. The RNA/DNA ratio value is thus proportional to the ratio of the DNA slope to the RNA slope, m_{DNA}/m_{RNA}:
   $(F_R/m_{RNA})/(F_D/m_{DNA}) = (F_R/F_D)*(m_{DNA}/m_{RNA})$
5. This offers an ability to standardize results from different studies to the same units by:
   a. Calculate a standardization factor (SF) by dividing the RNA and DNA standard curves slopes ratio of the reference protocol (P_{ref}) by the corresponding slope ratio of each particular protocol (P_i):

$$SF = (m_{RNA}/m_{DNA})_{Pref} / (m_{RNA}/m_{DNA})_{Pi}$$

   or rearranging: $SF = (m_{DNA}/m_{RNA})_{Pi} / (m_{DNA}/m_{RNA})_{Pref}$

   b. Divide the RNA/DNA values of each P_i protocol by the SF to yield a standardized RNA/DNA value (sRNA/DNA). sRNA/DNA values can then be directly compared to RNA/DNA values from P_{ref}.

We provide an example below using the results from this study.

	Individual standards		Common standards	
	standard curve slopes ratio m_{RNA}/m_{DNA}	SF	standard curve slopes ratio m_{RNA}/m_{DNA}	SF
P_{ref}	0.38	1.00	0.40	1.00
P_1	0.45	0.92	0.42	1.04
P_2	0.67	0.63	0.67	0.61
P_3	0.22	1.91	0.48	0.84
P_4	0.26	1.63	0.23	1.72

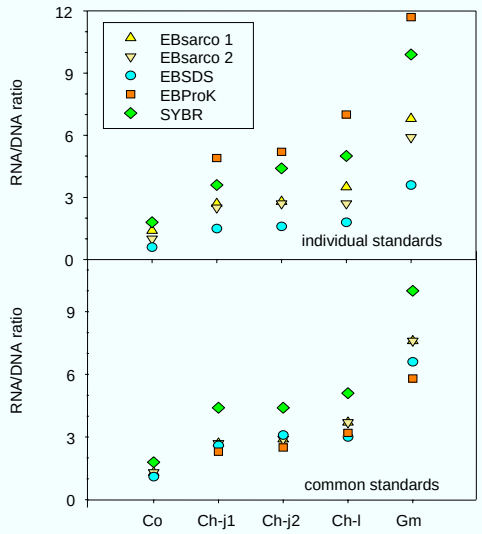
Results

1) The rank order of each tissue RNA/DNA within a laboratory was identical. However, the variance attributed to analytical protocol was 62%

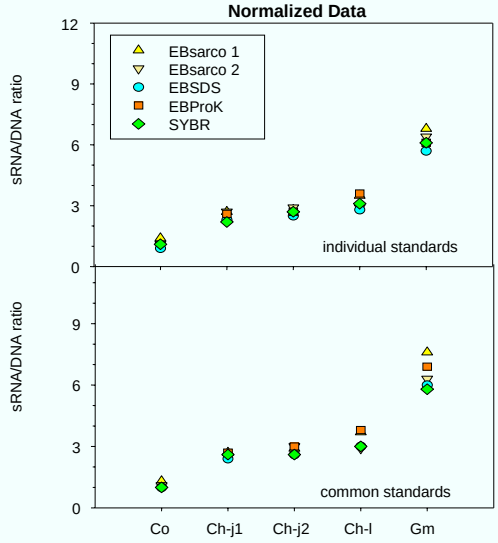
2) The use of common standards only reduced the variance attributed to analytical protocol to 46% of the total variance

3) Normalizing the RNA/DNA values with a procedure based on the ratio of the slopes of the standards reduced the variance attributed to analytical protocol to 5% of the total variance

Raw Data



Normalized Data



Variance components

	Individual standards		Common standards	
	R/D	sR/D	R/D	sR/D
Protocols within a sample type	62%	5%	46%	9%
All samples	38%	94%	53%	91%
Replicates within a protocol	<0.1%	<0.1%	<0.1%	<0.1%
Error	<1%	<1%	<1%	<1%

Conclusions

We propose that if the ratio of the slopes of the RNA and DNA standard curves is known, it should be possible to more meaningfully compare RNA/DNA values obtained in different laboratories using different spectrofluorometric methods for the analysis of nucleic acids in fish.

We recommend average standard curves be provided in future publications to facilitate such intercalibrations.

Literature

Berdalet E, Roldan C, Olivar MP (2005) Quantifying RNA and DNA in planktonic organisms with SYBR Green II and nucleases. Part B. Quantification in natural samples. Sci Mar 69 (1): 17-30

Caldarone EM, Buckley LJ (1991) Quantification of DNA and RNA in crude tissue extracts by flow injection analysis. Anal. Biochem. 199:137-141.