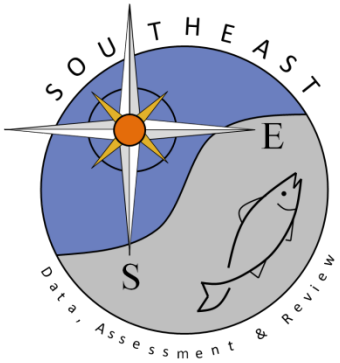
Blueline Tilefish Age Workshop II

Jennifer Potts, Andy Ostrowski, Adam Lytton, Kevin Spanik, Joey Ballenger, Eric Robillard, Katie Rogers, Michael Schmidtke, Naeem Willett, and Nikolai Klibansky

SEDAR50-DW18

Submitted: 9 December 2016



Please cite this document as:

Potts, J, A. Ostrowski, A. Lytton, K. Spanik, J. Ballenger, E. Robillard, Katie Rogers, Michael Schmidtke, Naeem Willett, and Nikolai Klibansky. 2016. Blueline Tilefish Age Workshop II. SEDAR50-DW18. SEDAR, North Charleston, SC. 36 pp.

Blueline Tilefish Age Workshop II

August 29-31, 2016

NMFS Beaufort Laboratory, Beaufort, NC

Participants and Affiliations:

Jennifer Potts – NMFS, SEFSC Beaufort, Host
Andy Ostrowski – NMFS, SEFSC Beaufort, Primary age reader
Adam Lytton – SCDNR, Primary age reader
Kevin Spanik – SCDNR, Primary age reader
Joey Ballenger – SCDNR
Eric Robillard – NMFS, NEFSC Woods Hole
Katie Rogers – NMFS, NEFSC Woods Hole, Primary age reader
Michael Schmidtke – ODU, Primary age reader (via webinar)
Naeem Willett – NMFS, SEFSC Panama City (via webinar)
Nikolai Klibansky – NMFS, SEFSC Beaufort, Rapporteur

Observers:

Erik Williams – NMFS, SEFSC Beaufort
Michelle Duval – NCDMF, SAFMC Rep, current chair of SAFMC
Julia Byrd – SEDAR Coordinator
Tracy McCulloch – NMFS, SEFSC Beaufort
Dalton Knight – NMFS, SEFSC Beaufort
Walt Rogers – NMFS, SEFSC Beaufort

The Blueline Tilefish (BLT) age workshop was convened to address the issue of inconsistency in age readings between three primary labs engaged in this work – NMFS Beaufort, SCDNR and ODU – and the need to include other fish ageing labs due to the extent of the blueline tilefish stock as determined in the stock ID workshop in June 2016. The goals of the workshop were to establish sagittal otolith sectioning methodology, set criteria for opaque zone interpretation, establish longevity of the species, estimate the amount of time to complete age readings for SEDAR50 and establish the rate of exchanges of samples between laboratories to ensure consistency in age readings.

Age Workshop I and SEDAR 32:

A review of the first BLT age workshop and then the subsequent work set the stage for why another workshop was needed. The first workshop was held in October 2012 with participants from NMFS Beaufort, SCDNR and ODU (Appendix A). The criteria for annulus interpretation was set based on expert opinion, though was not clearly defined. The workshop participants identified further research was needed to set annual zone interpretation. At the time of that workshop, SCDNR was not actively aging BLT, but were providing age data from Harris et al. (2004). Following the workshop, but prior to SEDAR32, a set of 280 samples were exchanged between the three labs. The results did raise concerns over consistency in age readings with average percent error (APE) values ranging from 11% - 27% (Table 1). Across the four independent "reads" of 271 individual fish available from this exchange, the APE was 25%. However, bias plots suggested no significant bias (Figure 1). SCDNR did not re-read the samples from the Harris et al. (2004) study for SEDAR 32. All of ODU and NMFS Beaufort age data were from

readings after the age workshop. All data at that point were used in SEDAR32 (SEDAR, 2013) to model population growth, but only data from NMFS Beaufort were used to characterize the fishery landings.

Some research recommendations regarding the ageing of BLT were recorded in the SEDAR32 Stock Assessment Report (SEDAR, 2013). They included the following.

1. Age readings of blueline tilefish need to be validated. Within and between lab variability in readings is large and needs to be addressed. The potential bias in age readings between laboratories also needs to be addressed with another age workshop and exchange of calibration sets of samples.
2. Marginal increment analysis needs to be undertaken in order to convert opaque zone counts to calendar ages. Samples processed and read in older studies will need to be re-examined and margin codes recorded for each.
3. An age error matrix needs to be used in the assessment model to account for differences and uncertainty in aging of Blueline Tilefish.
4. Implement a systematic age sampling program and systematic evaluation of aging error. Age samples were important in the assessment but reasonable samples were only available for the last 3-4 years of the assessment.
5. Given that this is an age-based assessment, the comparison and calibration studies for the age determination should receive high priority along with the marginal opaque zone analysis to determine if the opaque zone is formed annually.

One research recommendation, marginal increment analysis of the BLT otolith sections, was undertaken by Mike Schmidtke (ODU). He took radial measurements on the otolith sections from the core area to each opaque zone he considered to be an annulus and to the otolith margin in the ventral portion of the section at approximately a 45^0 angle. He found the analysis to be largely inconclusive and not reliable for converting counts to calendar ages.

Calibration Results – Pre-Age Workshop II:

January 2016 NMFS Beaufort Exchange

Due to concerns raised with the age readings from the three labs during SEDAR32 and changes in primary BLT age readers for SCDNR, the three labs exchanged calibration sets, one set from each lab, in preparation for SEDAR50. In January 2016 the first exchange was made between NMFS-Beaufort and SCDNR, with the primary intent at this time to train two new SCDNR age readers to read BLT. NMFS Beaufort slides (n = 299) were sent to SCDNR, with a subset of these slides being used to initially train the new SCDNR readers. Post initial training, the two SCDNR readers read these slides for a calibration read to determine if they were in agreement with the original NMFS Beaufort ages for these fish. Based on NMFS-Beaufort age determinations, 95% of the fish included in this calibration set possessed opaque zone count ≤10, with only opaque zone counts of 3, (n = 14), 4 (n = 26), 5 (n = 45), 6 (n = 78), 7 (n = 65), 8 (n = 33), 9 (n = 16), 10 (n = 5), 11 (n = 3), 14 (n = 2), and 21 (n = 2) being represented by multiple fish. Other opaque zone counts represented by the calibration set had only a single fish. Overall, the APE on 267 slides read by all three readers (NMFS-Beaufort recorded age, SCDNR Reader 1 and SCDNR Reader 2) was 14.9%. Pairwise comparisons of the readers resulted in APEs ranging from 6.9-16.2%, though confidence intervals of average difference suggested slight bias in age determinations (Table 2). Bias plots comparing the independent age reads can be found in Figure 2. The APE of the two independent age readers from SCDNR, as compared to the NMFS ages, was less than the target APE of 15% specified during the first BLT age workshop. Further, as SCDNR planned to perform consensus reads on any individuals for which independent reads differed between the readers during production aging, it was

felt that the final APE would likely fall between the extremes. Both labs went into production ageing at that point.

April 2016 Calibration Set Exchange

In April 2016 a second calibration set exchange was made between SCDNR and NMFS-Beaufort personnel. For this exchange, SCDNR slides (n = 184) were sent to NMFS Beaufort. The SCDNR historic readings of their set were based on the Harris et al. (2004) study. Simultaneously, the two newly trained SCDNR age readers re-read these slides as part of their production aging of Blueline Tilefish for SEDAR 50, getting individual reads for each reader and a consensus age if they disagreed. In contrast to the NMFS-Beaufort calibration set previously exchanged, only 55% of this set was composed of fish possessing opaque zone counts ≤ 10, with 16% representing fish with opaque zone counts ≥ 20. Overall, the APE on 168 slides read by three readers (SCDNR historic age, SCDNR Consensus, and NMFS-Beaufort) was 27.6%. Pairwise comparisons of contemporary SCDNR reads with the historic SCDNR opaque zone counts resulted in APEs ranging from 11.3-14.8%, average differences in age of less than 1 year, and with >90% absolute agreement by ±7 increments (Table 3). Pairwise comparisons of contemporary NMFS-Beaufort opaque zone counts compared to either the historic or contemporary consensus opaque zone counts made by SCDNR resulted in APEs ranging from 31.5-32.2%, average opaque zone count differences of 5, and with <90% absolute agreement at ±10 increments (Table 3). Bias plots comparing the independent age reads can be found in Figure 3. These results suggested non-biased age reads between reads deriving from the SCDNR, though still relatively imprecise. Comparisons with the NMFS opaque zone counts suggested a large degree of bias, with the degree of bias monotonically increasing with opaque zone count (Figure 3D and Figure 3E).

May 2016 Calibration Set Exchange

In May 2016, a third exchange was made. The same set of ODU slides (n = 100) were sent to NMFS-Beaufort and SCDNR. Based on NMFS-Beaufort age determinations, 82% of the fish included in this calibration set possessed opaque zone counts ≤10, with only opaque zone counts of 3 (n = 2), 4 (n = 6), 5 (n = 5), 6 (n = 19), 7 (n = 23), 8 (n = 15), 9 (n = 7), 10 (n = 4), 11 (n = 2), 12 (n = 7), 13 (n = 2), 14 (n =2) and 15 (n = 3) being represented by multiple fish. Comparing SCDNR age readers to the ODU calibration set ages, APE estimates ranged from 9.4-10.6%, average opaque zone count differences of 0.13 – 0.16 years, with percent agreement exceeding 90% by ±3 increments (Table 4). A pairwise comparison of the NMFS-Beaufort age reader to the ODU calibration set opaque zone counts resulted in an APE of 18.0%, an average difference in opaque zone counts of 2 increments, and percent agreement in excess of 90% at ±5 increments (Table 4). Bias plots comparing the independent age reads to the ODU calibration set opaque zone counts can be found in Figure 4. As with the exchange in May, these results suggested non-biased age reads deriving from the SCDNR, though still relatively imprecise. Comparisons with the NMFS opaque zone counts suggested a large degree of bias, with the degree of bias monotonically increasing with opaque zone count (Figure 4C).

Bomb Radiocarbon Analysis:

Results of the calibration set exchanges detailed above that occurred post-SEDAR 32 but prior to SEDAR 50 prompted SCDNR to prepare otoliths for bomb radiocarbon analysis as a means of validating the age readings. Measuring levels of bomb radiocarbon in fish otoliths is generally considered a reliable means of validating the ages of fish within 1-2 years. SCDNR identified 40 otoliths from their collection to process for radiocarbon readings. They extracted the core area of the sagittal otoliths and sent them to National Ocean Services Accelerator Mass Spectrometry (NOSAMS) laboratory for testing. The results were analyzed using $F^{14}C$ values from these BLT otoliths compared to reference values from the South Atlantic Bight (SAB), Gulf of Mexico (GOM), northwest Atlantic (NWA) and Gulf of Alaska (GOA).

The $F^{14}C$ values were plotted against the presumed birth year for each fish in the sample (Figure 5). The birth year of each fish was determined by subtracting the SCDNR consensus age reading from the year of capture. These values were also plotted against the reference chronologies as listed above (Figure 6). The uptake pattern of $F^{14}C$ in adult Blueline Tilefish captured off the coast of South Carolina, based on the aging methodology of SCDNR readers, match expectations from other species and areas (e.g. Baker and Wilson 2001, Campana et al. 2008, Kastelle et al. 2008, Lytton et al. 2016, Piner and Wischniowsi 2004). The chronology indicates a period of rapid increase in otolith core $F^{14}C$ over an approximately 10-15 year period from the early-1960s through the mid-1970s (Figure 5). The similarity in length of uptake period suggest that increments, as identified using the current aging methodology, are deposited on an annual basis. Had the aging methodology not been identifying structures formed annually there would have been less correspondence in length of uptake period in the current study to published reference chronologies. Longer periods of rapid increase, relative to reference chronologies, are indicative of more than a single opaque zone forming annually, while shorter periods represent the converse. If the aging methodology had not been successful at identifying consistent structures, there would have been no observable uptake pattern for Blueline Tilefish.

Despite the observance of the expected uptake pattern of ^{14}C in the otolith cores of Blueline Tilefish, results were inconclusive regarding the degree of aging bias that may be present using the SCDNR aging methodology. Degree of aging bias varied depending on the reference chronology the Blueline Tilefish $F^{14}C$ values were compared too. When compared to a $F^{14}C$ reference chronology composed of unknown age Wreckfish, Red Bream, and Barrelfish from the Charleston Bump, the best estimate of Blueline Tilefish aging bias was 3-4%, with a 95% confidence interval of -4 to 16% (Table 5 and Table 6). In contrast, when compared to the GOM, NWA, and GOA reference chronologies, the *h*-statistic suggests the best estimates of aging bias is -37%, -26%, and -22%, respectively (Table 5). Confidence intervals for proportional aging bias were -38% to -32%, -30% to -15%, and -27% to -19% when compared to the GOM, NWA, and GOA reference chronologies, respectively (Table 6). Generally, the results suggest that SCDNR readings are either unbiased or biased young.

Exchange of NMFS-Beaufort Samples with SCDNR

Prior to the workshop, a final exchange of 895 samples recently aged by NMFS-Beaufort personnel were exchanged with SCDNR. These samples represented a random assortment of fishery-dependent samples collected by NMFS-Beaufort during the years 2013-2015. Based on NMFS-Beaufort opaque zone counts, 87% of these samples represented fish with opaque zone counts ≤10, with only 1% representing fish with opaque zone counts ≥20. The two SCDNR age readers independently assigned ages to all fish included in this exchange. Pairwise comparisons of the two independent SCDNR reads resulted in an APE of 12%, with an average difference of opaque zone counts of 0, and with >90% absolute agreement by ±5 increments (Table 7). Bias plots possessed little indication of systematic aging bias between the two SCDNR readers (Figure 7). Based on SCDNR ages, 56-59% of these samples represented fish with opaque zone counts ≤10, with 8-10% representing fish with opaque zone counts ≥20. In contrast, pairwise comparisons of the independent SCDNR reads compared to the NMFS-Beaufort opaque zone counts results in an APE of 26%, with an average difference of opaque zone counts of 4 years, and with >90% absolute agreement not occurring until ±10. Bias plots continued to suggest a monotonically increasing degree of aging bias with opaque zone count between SCDNR and NMFS-Beaufort opaque zone counts (Figure 7)

Aging Workshop

In light of the above information, the 2nd Blueline Tilefish age workshop was convened on August 29-31, 2016. The workshop was devoted to identifying best methods for sectioning the BLT

otoliths for age reading and interpretation of the otolith structure. Following is a summary of the key findings and recommendations.

Methods for Sectioning BLT Otoliths

In general, methods for sectioning BLT otoliths as established during the first BLT age workshop should be followed. The otoliths need to be embedded in epoxy to stabilize them during the cutting process. Multiple sections from each otolith surrounding the core area should be taken with a single blade/multiple cuts or a multi-blade set up with spacers on a low-speed saw (e.g., Isomet). The sections should be 0.40 mm thickness. The serial sections are adhered to a glass slide with a clear adhesive or mounting medium and then covered with a liquid mounting medium (e.g., DePex (GURR), Flo-texx, Cytoseal). Details of the sectioning process by NMFS Beaufort is included in Appendix B (Ostrowski, 2016).

Interpretation of otolith structure

Once sectioning methodology was agreed upon, the workshop discussion turned to interpretation of the structure of the BLT otolith sections. Each lab actively ageing BLT had taken pictures of the otolith sections and annotated them with markers on the opaque zones they were counting. Much discussion was had about which side of the section each person preferred to take their readings. The SCDNR age readers generally counted opaque zones on the dorsal side of the otolith; conversely, the age readers from NMFS-Beaufort and ODU tended to make counts on the ventral side. All groups tried to use both sides to verify that the opaque zones could be seen on both. For the NMFS-Beaufort and ODU readers, if counts differed on the ventral side compared to dorsal side, the ventral side counts were usually recorded. For the SCDNR age readers, if counts differed, the dorsal side counts were usually recorded. All readers did use different planes of the otolith to count on, but one comment made was that counts tended to be higher if made along the sulcal groove. The inner increments (up to ~ 6 or 7) were found to be broad and diffuse, thus all age readers counted these wide fields that may encompass several opaque bands as a single increment. The outer increments (>6 or 7) became more regularly spaced, but were tightly grouped.

The interpretation of the structure lead to discussion of what magnification to use when reading the sections. The NMFS-Beaufort reader tended to use the same magnification for all readings (~20x). SCDNR readers found that inner increments (i.e., near the otolith core) were more clearly defined at 40-60X magnification, while outer increments were best read at 60-100X magnification. The ODU age reader also suggested that the slides could be tilted and the opaque zones may become more distinct.

All participants in the workshop looked at projected images of the BLT sections processed by the three labs, but there seemed to be no consistency across the individual labs with interpretation of the otolith structure. Differences emerged regarding what individual readers were counting as increments. Comments were made regarding the inconsistency in spacing of the first 6 or 7 opaque zones, such as the first 3 zones were relatively close together and then there was a large gap before the 4^{th} zone or a relatively large gap between the 4^{th} and 5^{th} zone. Questions were raised as to all of the finely spaced opaque zones should be read individually and when they should be grouped together as one annulus. No one seemed to have a solution to the questions or comments. Everyone felt that the 2-D images were difficult to interpret and did not represent what a reader would have seen through the microscope. The group decided to look at the physical samples randomly selected from the NMFS Beaufort set and the SCDNR set, because the reader can manipulate the lighting, magnification and tilt of the slide as well as focus through the sample (depth of field) when working on a microscope.

After the age readers from each lab looked at 40 samples, the interpretation of the otolith structure was not resolvable as no consensus could be developed across all labs regarding otolith

structure interpretation. Each reader seemed to have the results of the bomb radiocarbon study in mind when reading the samples, thus the opaque zone counts tended to be higher than the initial readings. Further, even when not higher, individual age reads for most ages were not precise. Some of the readings differed by only 3, but others differed by as much as 12. The readings by the three most experienced BLT agers in the group, NMFS Beaufort Reader1 and the SCDNR Reader1 and Reader2, seemed to be coming into better agreement, but their counts were still different from the original readings and precision of age reads became a concern. When the sections were projected, though the counts were similar for a particular sample, the age readers did not agree on what structure they were counting. Overall the group agreed that producing precise ages of BLT was not attainable during the workshop.

Workshop Conclusions and Research Recommendations

The consensus of the participants of the workshop is that Blueline Tilefish could not be precisely aged at this time. At this point we have not achieved a high degree of precision. Though we have some indication of the relative accuracy of the age readings, on average, based on the bomb radiocarbon study (i.e., accurate to under-ages; no indication of over-aging), the lack of precision means inclusion of the age data in a stock assessment may not allow for tracking of cohorts in the age compositions. This would have deleterious effects to the assessment, and lack of ability to track cohorts was a major reason why age compositions for Blueline Tilefish were down weighted during SEDAR 32.

Research recommendations include

1. A reference chronology of bomb radiocarbon for the U.S. South Atlantic area needs to be established using known-age or unknown-age fish for which an aging methodology has been validated. Particular emphasis should be to obtain a reference chronology for deep water areas (>250 m), as age determination of most deep water fish species in the region is difficult.
2. Explore the use of other aging methodologies and structures for ageing Blueline Tilefish.
3. Identify more BLT otolith samples, for example from the West Florida Shelf (GOM), for bomb radiocarbon analyses.

Literature Cited

Baker, M.S. and C.A. Wilson. 2001. Use of bomb radiocarbon to validate otolith section ages of Red Snapper *Lutjanus campechanus* from the northern Gulf of Mexico. Limnol. Oceanogr. 46: 1819-1824.

Campana, S.E., J.M. Casselman, and C.M. Jones. 2008. Bomb radiocarbon chronologies in the Arctic, with implications for the age validation of Lake Trout (*Salvelinus namaycush*) and other Arctic species. Can. J. Fish. Aquat. Sci. 65(4): 733-743.

Francis, R.I.C.C., S.E. Campana, and H.L. Neil. 2010. Validation of fish ageing methods should involve bias estimation rather than hypothesis testing: a proposed approach for bomb radiocarbon validations. Can. J. Fish. Aquat. Sci. 67: 1398-1408.

Harris, PJ, D.M. Wyanski, and P. T. Powers Mikell. 2004. Age, Growth, and Reproductive Biology of Blueline Tilefish along the Southeastern Coast of the United States, 1982–1999. Trans. Amer. Fish. Soc. 133(5):1190-1204.

Kastelle, C.R., D.K. Kimura, and B.J. Goetz. 2008. Bomb radiocarbon validation of Pacific Ocean Perch (*Sebastes alutus*) using new statistical methods. Can. J. Fish. Aquat. Sci. 65: 1101-1112.

Lytton, A.R., J.C. Ballenger, M.J.M. Reichert, and T.I. Smart. 2016. Age validation of the north Atlantic stock for Wreckfish (*Polyprion americanus*), based on bomb radiocarbon (^{14}C), and new estimates of life history parameters. Fish. Bull. 114: 77-88.

Ostrowski, A. 2016. Standard Operative Procedure for Embedding and Sectioning Blueline Tilefish (*Caulolatilus microps*). SEDAR50-DW08. SEDAR, North Charleston, SC. 10 pp.

Piner, K.R. and S.G. Wischinowski. 2004 Pacific Halibut chronology of bomb radiocarbon in otoliths from 1944 to 1981 and a validation of ageing methods. J. Fish Biol. 64: 1060-1071.

SEDAR. 2013. SEDAR 32 – South Atlantic blueline tilefish Stock Assessment Report. SEDAR, North Charleston SC. 378 pp.

Table 1: Results of inter-laboratory calibration study conducted after the 1st Blueline Tilefish age workshop and before SEDAR 32. In total, ages from four independent "readers" were available for investigation of aging precision and bias among sets of readers. The four "readers" included consensus age reads available from the South Carolina Department of Natural Resources (SCDNR), consensus age reads from Old Dominion University (ODU), and independent age reads from two readers at the NOAA Fisheries Southeast Fisheries Science Center, Beaufort Lab (NMFS-1, NMFS-2). APE = average percent error; Avg = average age difference; LCI and UCI = lower and upper 95% confidence interval about difference.

				Difference		Percent Agreement						
Reader 1	**Reader 2**	**n**	**APE**	**Avg**	**CI**	**±0**	**±1**	**±2**	**±3**	**±5**	**±7**	**±10**
ODU	SCDNR	281	12.49	-1.61	-2.04- -1.18	12.46	38.08	56.94	72.24	88.96	9.24	99.64
NMFS-1	SCDNR	275	20.9	-2.86	-3.35- -2.37	6.18	20.36	41.1	57.82	81.82	90.9	99.27
NMFS-1	ODU	275	27.02	-4.30	-4.64- -3.96	4	10.18	19.27	35.27	66.55	90.18	99.64
NMFS-1	NMFS-2	271	10.78	-0.30	-0.7-0.09	28.04	61.99	78.97	86.35	93.36	96.31	98.3
NMFS-2	SCDNR	276	19.6	-2.46	-2.97- -1.95	7.97	26.09	42.39	59.78	84.06	92.03	98.91
NMFS-2	ODU	276	25.35	-4.0	-4.32- -3.62	1.45	9.06	21.74	32.25	70.29	90.94	99.64

Table 2: Results of SCDNR calibration reads of NMFS-Beaufort calibration set completed in January of 2016. This exchange was conducted prior to initiation of production aging by SCDNR for SEDAR 50 and was used to assess the successful implementation of the aging methodology described during the first Blueline Tilefish age workshop held in 2012 by two new SCDNR age readers. The 2012 Blueline Tilefish age workshop set a target of less than a 15% APE for independent age-reads on calibration sets.

				Difference		Percent Agreement						
Reader 1	**Reader 2**	**n**	**APE**	**Avg**	**CI**	**±0**	**±1**	**±2**	**±3**	**±5**	**±7**	**±10**
NMFS	SCDNR-1	269	6.86	-0.48	-0.68 - -0.29	47.58	80.30	88.10	93.68	97.77	99.63	100.00
NMFS	SCDNR-2	291	14.34	0.57	0.33 - 0.81	21.99	56.01	80.07	92.10	98.63	99.31	100.00
SCDNR-1	SCDNR-2	266	16.23	0.94	0.65 - 1.23	16.54	51.50	73.68	85.34	96.24	98.12	100.00

Table 3: Results of SCDNR and NMFS calibration reads of the historic SCDNR calibration set completed in April of 2016. This exchange occurred during production aging of Blueline Tilefish for SEDAR 50 by both the NMFS-Beaufort and SCDNR aging laboratories. Historic – historic opaque zone counts for Blueline Tilefish as presented in Harris et al. (2004); SCDNR-1 – contemporary opaque zone counts made by SCDNR Reader 1; SCDNR-2 – contemporary opaque zone counts made by SCDNR Reader 2; Consensus – contemporary opaque zone counts based on consensus ages by SCDNR readers; NMFS – contemporary opaque zone counts made by the NMFS-Beaufort reader.

				Difference		Percent Agreement						
Reader 1	**Reader 2**	**n**	**APE**	**Avg**	**CI**	**±0**	**±1**	**±2**	**±3**	**±5**	**±7**	**±10**
Historic	SCDNR-1	181	12.31	0.12	-0.65 - 0.88	19.89	45.86	62.43	73.48	83.98	91.71	94.48
Historic	SCDNR-2	169	14.77	0.40	-0.32 - 1.13	10.06	41.42	55.62	68.05	85.21	91.72	95.27
Historic	Consensus	168	11.32	-0.51	-1.11 - 0.10	17.26	48.21	64.29	77.98	88.69	93.45	97.02
Historic	NMFS	184	31.48	4.84	3.79 - 5.88	3.80	13.59	27.17	38.59	61.41	69.57	83.15
Consensus	NMFS	168	32.17	5.05	4.09 - 6.01	4.17	12.50	22.62	35.71	57.74	75.60	84.52

Table 4: Results of SCDNR and NMFS calibration reads of the ODU calibration set in May 2016. This exchange occurred during production aging of Blueline Tilefish for SEDAR 50 by both the NMFS-Beaufort and SCDNR aging laboratories. ODU – opaque zone counts for the ODU calibration set; SCDNR-1 – contemporary opaque zone counts made by SCDNR Reader 1; SCDNR-2 – contemporary opaque zone counts made by SCDNR Reader 2; NMFS – contemporary opaque zone counts made by the NMFS-Beaufort reader.

				Difference		Percent Agreement						
Reader 1	**Reader 2**	**n**	**APE**	**Avg**	**CI**	**±0**	**±1**	**±2**	**±3**	**±5**	**±7**	**±10**
ODU	SCDNR-1	100	9.42	0.13	-0.25 - 0.51	28.00	63.00	85.00	92.00	98.00	100.00	100.00
ODU	SCDNR-2	92	10.60	0.16	-0.24 - 0.57	19.57	56.52	83.70	92.39	98.91	100.00	100.00
ODU	NMFS	100	17.98	2.30	2.03 - 2.57	5.00	29.00	61.00	80.00	98.00	100.00	100.00

Table 5: The effect of assumed bias on the statistic h*, which measures the extent to which the Blueline Tilefish test data tends to be to the left ($h < 0$) or right ($h > 0$) of a given reference collection. Gray shaded cells indicate the degree of proportional bias between which the* h*-statistic goes from negative to positive; the best estimate of age bias, relative to a given reference set, is between this interval. See Francis et al. (2010) for details regarding methodology.*

	Assumed Proportional Bias (%)												
Ref.	**-40**	**-35**	**-30**	**-25**	**-20**	**-15**	**-10**	**-5**	**0**	**+5**	**+10**	**+15**	**+20**
SAB	-11.3	-9.48	-7.67	-5.65	-4.36	-3.37	-1.84	-1.24	-0.54	0.2	0.88	1.45	1.95
GOM	-1.58	0.61	1.9	3.05	3.96	4.87	5.78	6.47	7.36	8.4	9.03	9.46	10.2
NWA	-3.86	-2.14	-1.04	0.51	1.09	2.11	2.67	3.56	4.46	5.4	5.91	6.24	7.08
GOA	-4.98	-3.03	-1.71	-0.4	0.47	1.55	2.19	3.08	4.18	5.3	5.43	6.41	7.39

Table 6: Results of age bias estimation comparing the observed Blueline Tilefish $F^{14}C$ chronology to the four finfish reference collections considered. The minimum and maximum year for which both the Blueline Tilefish and a given reference chronology were considered increasing is provided. Also provide is the minimum and maximum $F^{14}C$ estimates observed in a given reference chronology over the restricted year range. Results of 10,000 simulations indicate that random error alone could have resulted in the given 95% confidence interval for the h*-statistic assuming a given reference chronology is assumed the "true" pattern. Via interpolation, the 95% confidence interval for age bias is provided relative to each reference chronology.*

	Year		$F^{14}C$		*h* CI		Age Bias CI	
Reference	**Min**	**Max**	**Min**	**Max**	**Lower**	**Upper**	**Lower**	**Upper**
SAB Reference	1962	1975	0.9109	1.1044	-0.7018	1.4752	-4	16
GOM Reference	1960	1972	0.9493	1.1515	-0.5101	1.0351	-38	-32
NWA Reference	1960	1970	0.9722	1.0680	-0.6342	1.7199	-30	-15
GOA Reference	1960	1970	0.91619	1.1096	-0.6334	0.6931	-27	-19

Table 7: Results of SCDNR reads of 895 NMFS-Beaufort fishery-dependent Blueline Tilefish samples collected from 2013-2015. This exchange occurred post-production aging of Blueline Tilefish for SEDAR 50 by both the NMFS-Beaufort and SCDNR aging laboratories. NMFS – opaque zone counts for the samples as estimated by the NMFS-Beaufort age reader; SCDNR-1 – opaque zone counts made by SCDNR Reader 1; SCDNR-2 – opaque zone counts made by SCDNR Reader 2.

				Difference		Percent Agreement						
Reader 1	**Reader 2**	**n**	**APE**	**Avg**	**CI**	**±0**	**±1**	**±2**	**±3**	**±5**	**±7**	**±10**
NMFS	SCDNR-1	874	25.50	-4.38	-4.66 - -4.11	6.41	19.34	35.93	49.43	69.91	83.30	92.45
NMFS	SCDNR-2	653	26.36	-4.44	-4.71 - -4.17	7.04	19.91	30.63	44.10	68.15	81.93	94.18
SCDNR-1	SCDNR-2	646	11.80	0.42	0.14 - 0.69	18.27	45.82	61.61	76.78	90.25	95.98	98.61

Figures

Figure 1: Paired readings of Blueline Tilefish age samples (n = 280) by NMFS-Beaufort, SCDNR, and ODU as presented to SEDAR32. The blue line is considered true age by reader on x-axis. The red line is the average age reading compared to the "true age" by reader on y-axis. Error bars are 95% confidence intervals.

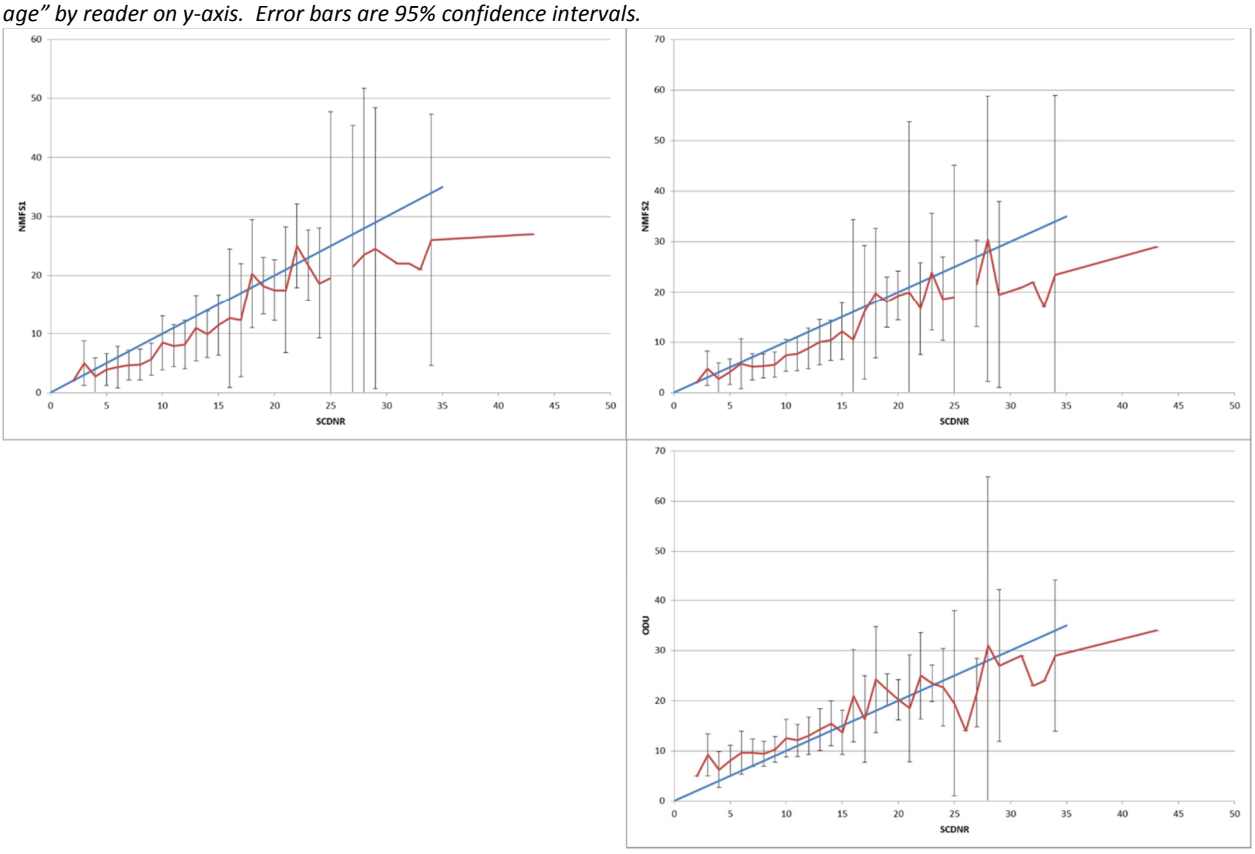
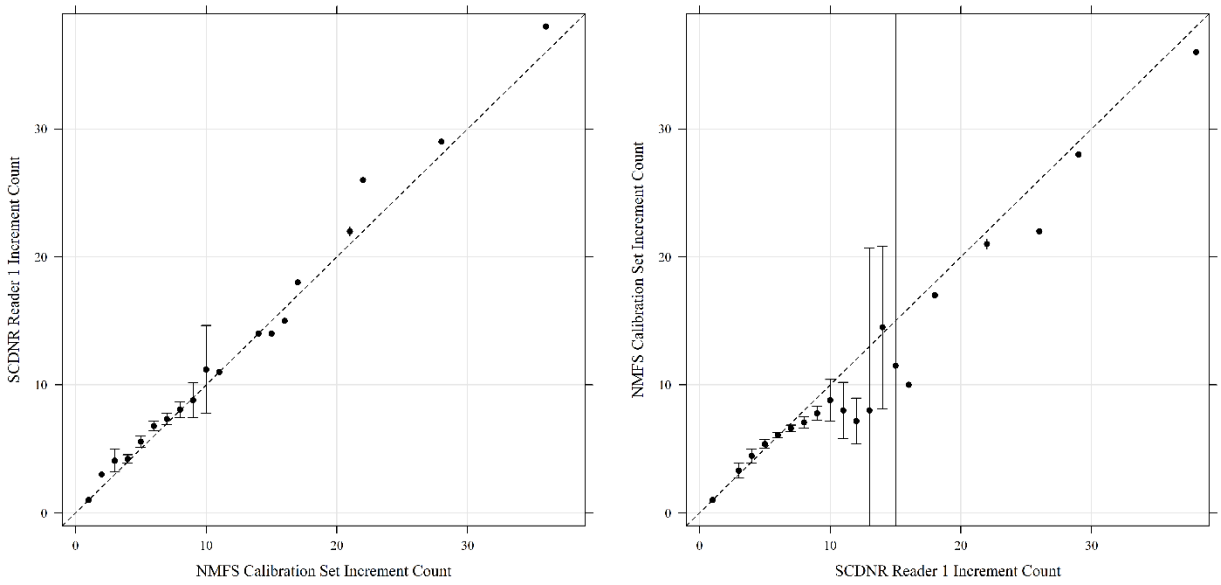
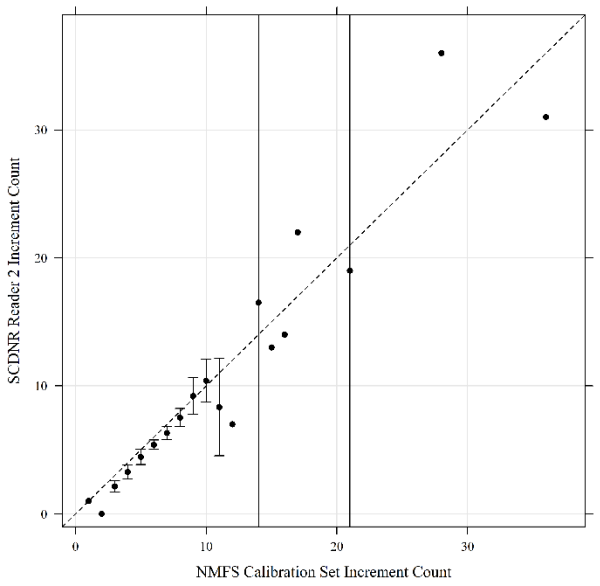


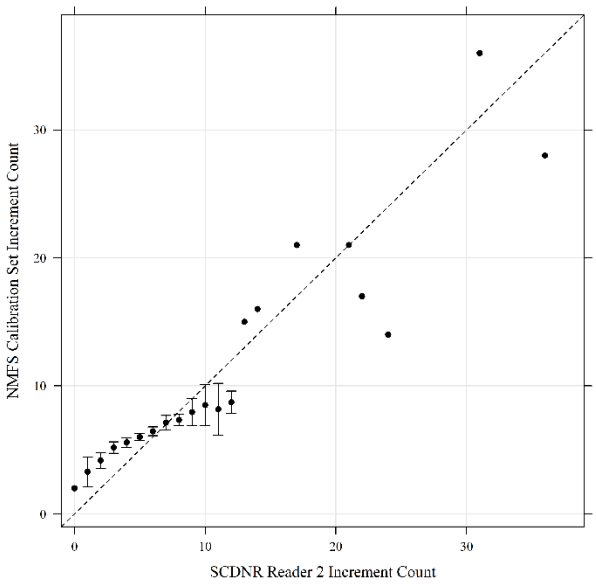
Figure 2. Results of paired readings of Blueline Tilefish from an exchange of a NMFS-Beaufort calibration set in January 2016 with the SCDNR (see Table 2). This exchange was conducted to facilitate training of two new SCDNR age readers for Blueline Tilefish and to assess inter-laboratory calibration. The three panels represent, A) comparison of NMFS-Beaufort opaque zone counts to SCDNR Reader 1 opaque zone counts, B) comparison of NMFS-Beaufort opaque zone counts to SCDNR Reader 2 opaque zone counts, and C) SCDNR Reader 1 opaque zone counts to SCDNR Reader 2 opaque zone counts. Note, based on NMFS-Beaufort ages, approximately 5% (n = 16) of this calibration set represents Blueline Tilefish older than age 10. The dashed line is considered "true age" by reader on x-axis. The black dots are the average age reading compared to the "true age" by reader on y-axis. Error bars are 95% confidence intervals.

A)



B)





C)

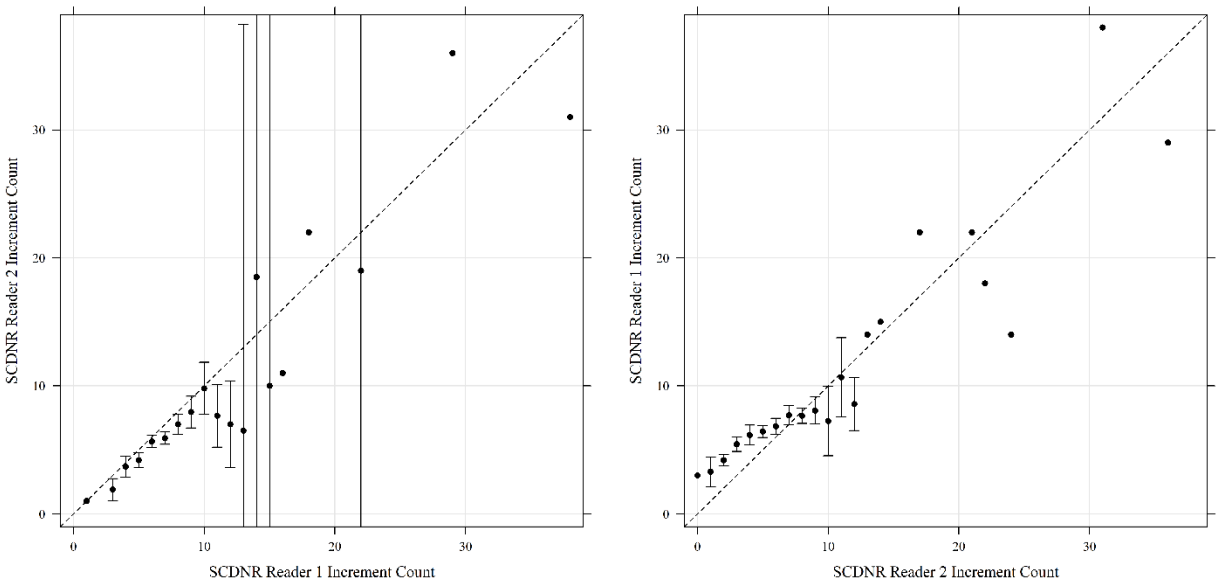
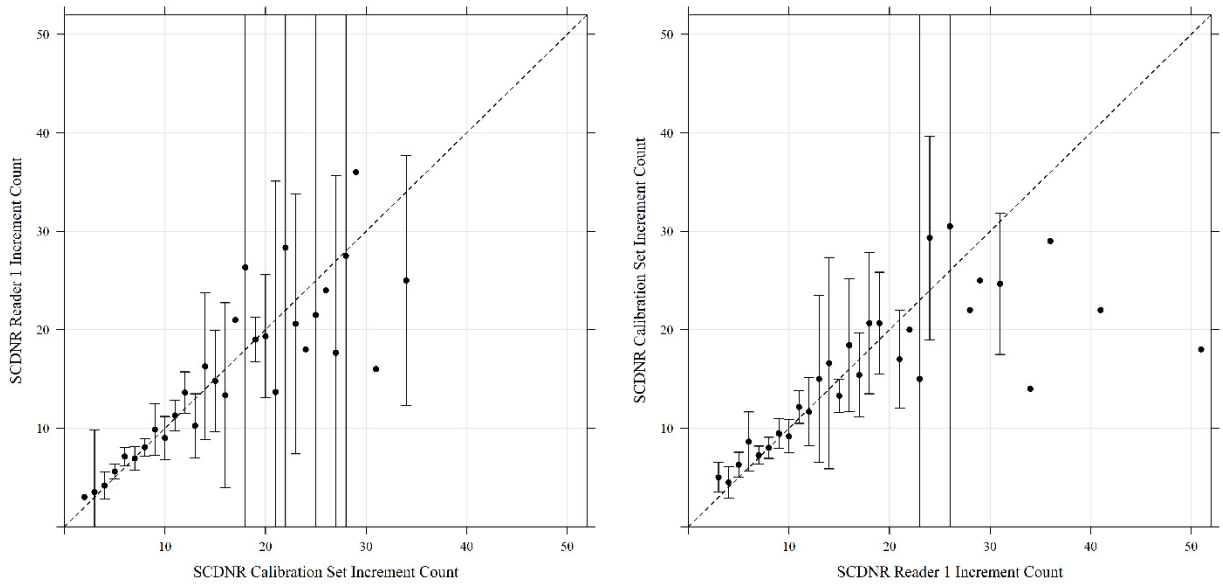
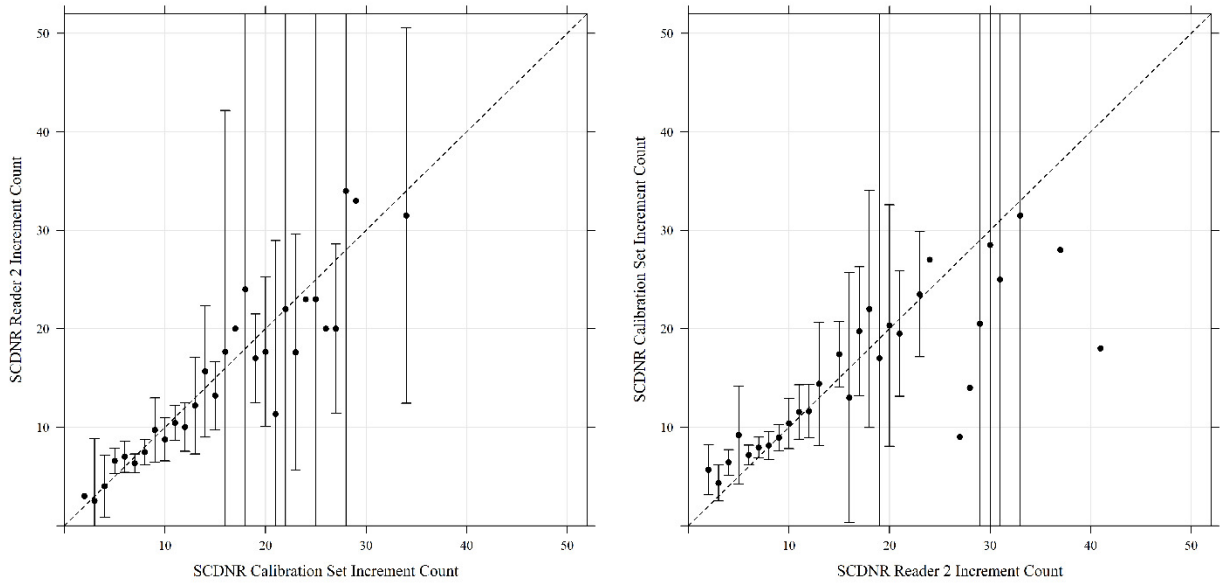

SCDNR Reader 2 Increment Count
SCDNR Reader 1 Increment Count
0
10
20
30
SCDNR Reader 1 Increment Count
SCDNR Reader 2 Increment Count

Figure 3. Results of paired readings of Blueline Tilefish from an exchange of a SCDNR calibration set in April 2016 with the SCDNR (see Table 3). The panels represent, A) comparison of opaque zone counts from SCDNR Reader 1 and the SCDNR calibration set, B) comparison of opaque zone counts from SCDNR Reader 2 and the SCDNR calibration set, C) comparison of opaque zone counts from SCDNR Consensus and the SCDNR calibration set, D) comparison of opaque zone counts from NMFS and the SCDNR calibration set, and E) comparison of opaque zone counts from NMFS and the SCDNR Consensus. The dashed line is considered "true age" by reader on x-axis. The black dots are the average age reading compared to the "true age" by reader on y-axis. Error bars are 95% confidence intervals.

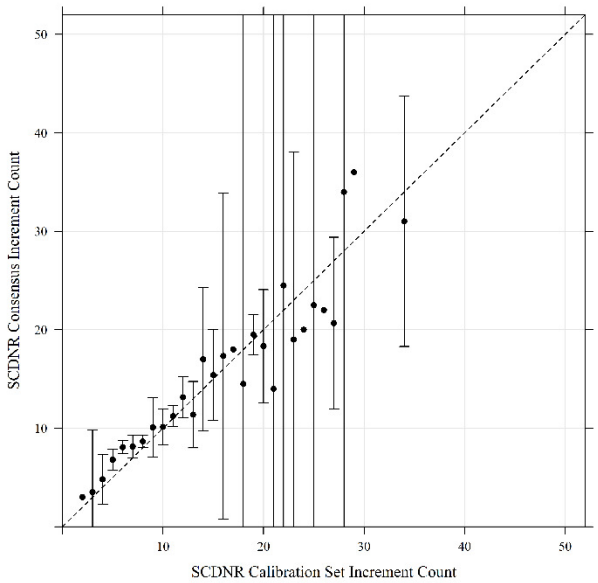
A)

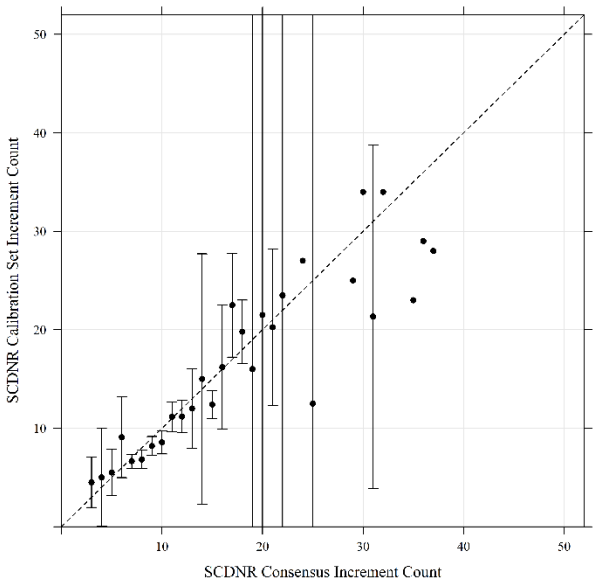


B)

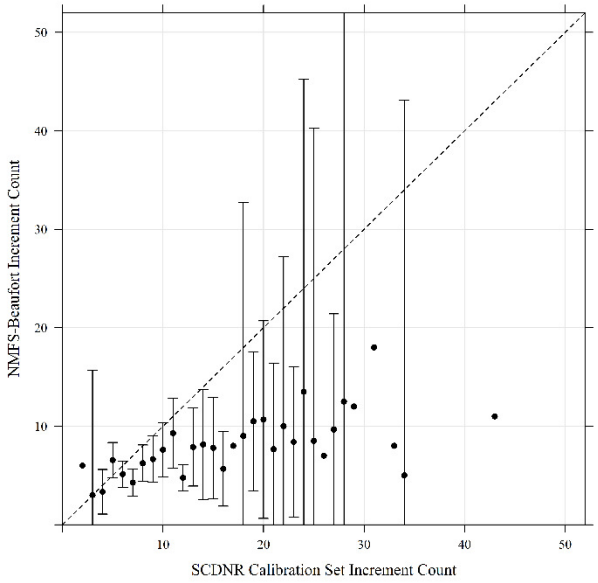


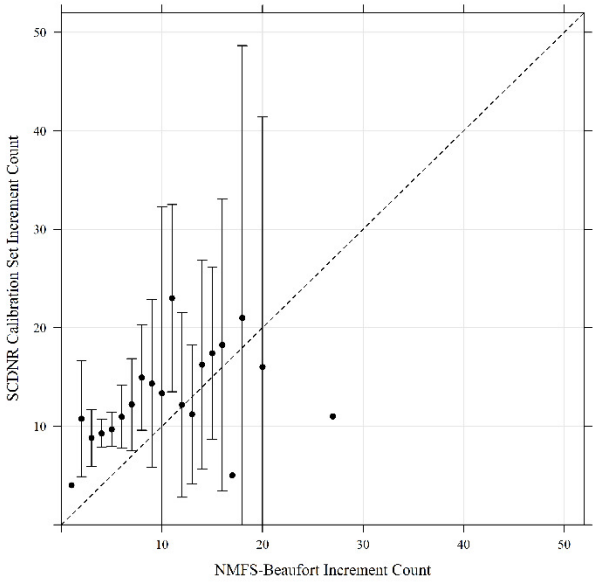
C)


SCDNR Consensus Increment Count
SCDNR Calibration Set Increment Count
10
20
30
40
50

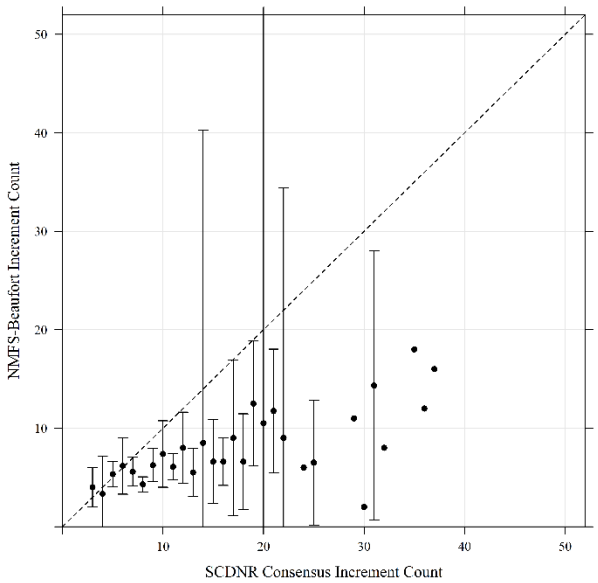

SCDNR Calibration Set Increment Count
SCDNR Consensus Increment Count
10
20
30
40
50

D)


NMFS-Beaufort Increment Count
SCDNR Calibration Set Increment Count
10
20
30
40
50


SCDNR Calibration Set Increment Count
NMFS-Beaufort Increment Count
10
20
30
40
50

E)


NMFS-Beaufort Increment Count
SCDNR Consensus Increment Count
10
20
30
40
50

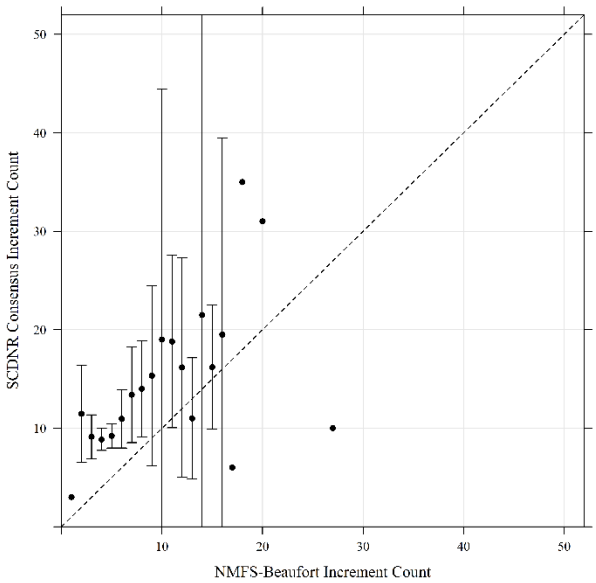
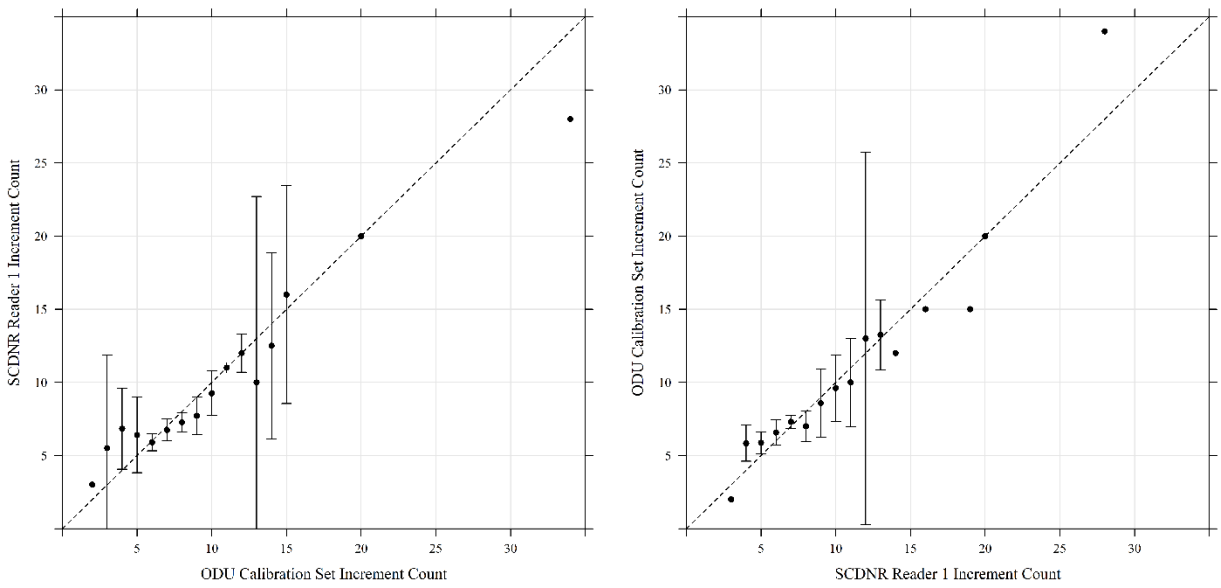
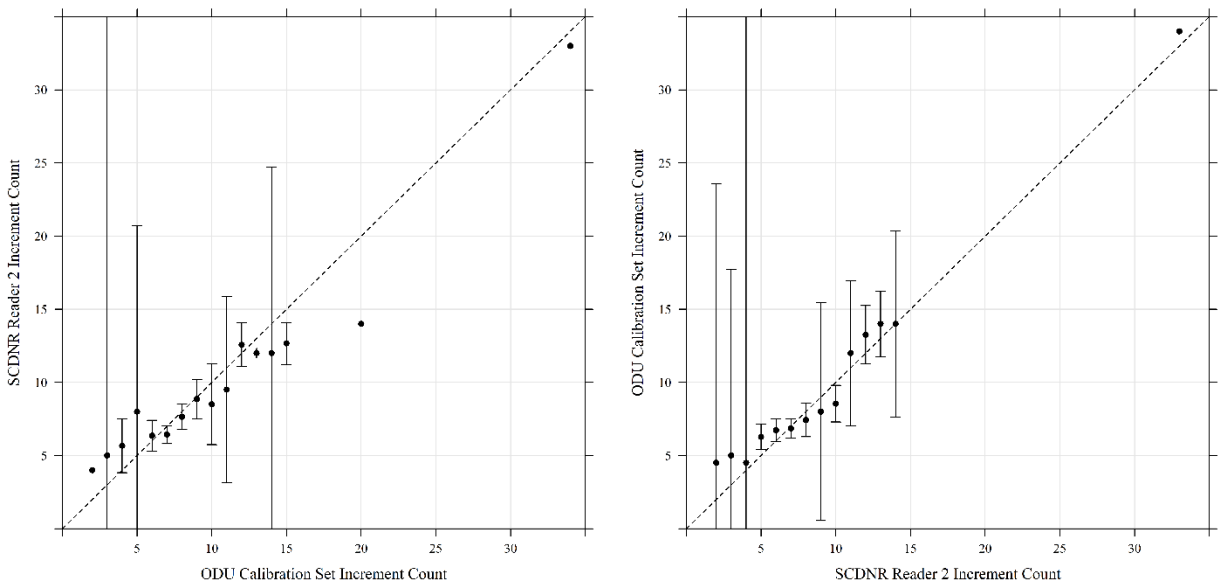

SCDNR Consensus Increment Count
NMFS-Beaufort Increment Count
10
20
30
40
50

Figure 4. Results of paired readings of Blueline Tilefish from an exchange of an ODU calibration set in May 2016 (see Table 4). The three panels represent, A) a comparison of SCDNR Reader 1 opaque zone counts to ODU calibration set opaque zone counts, B) a comparison of SCDNR Reader 2 opaque zone counts to ODU calibration set opaque zone counts, and C) a comparison of NMFS-Beaufort reader opaque zone counts to ODU calibration set opaque zone counts. The dashed line is considered "true age" by reader on x-axis. The black dots are the average age reading compared to the "true age" by reader on y-axis. Error bars are 95% confidence intervals.

A)



B)



C)

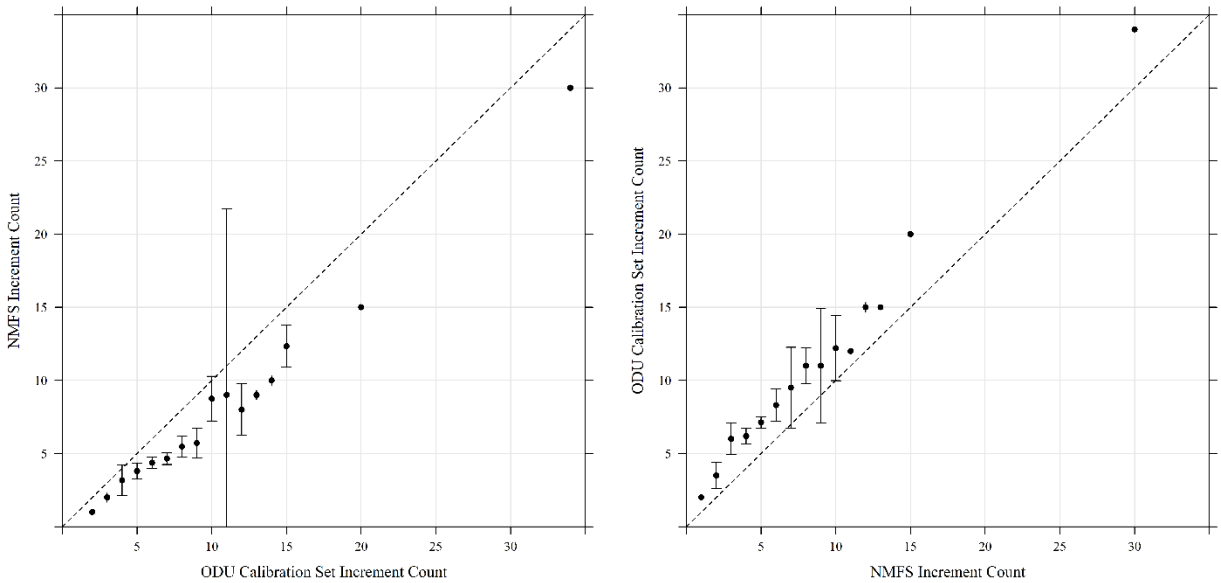


Figure 5: Observed $F^{14}C$ versus year class (a.k.a., birth year) for Blueline Tilefish based on SCDNR age readings. Horizontal and vertical error bars represent the standard error estimate for age estimates and the $F^{14}C$ σ from the bomb radiocarbon analysis, respectively

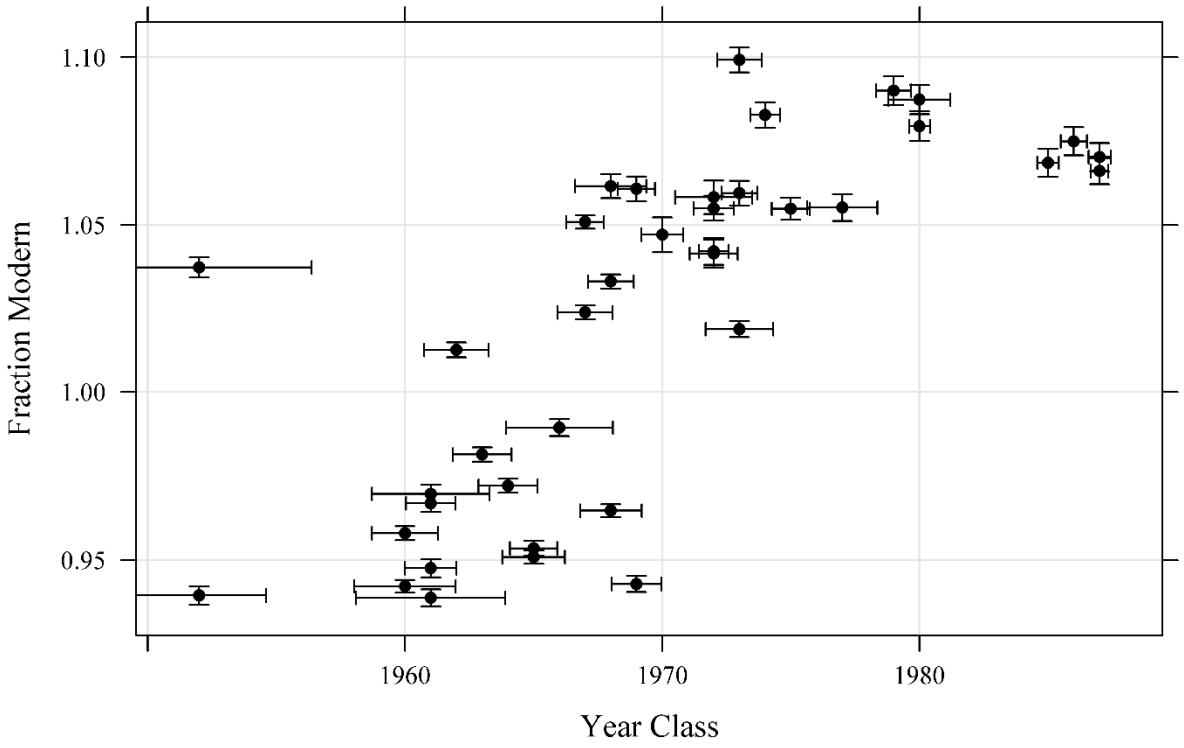


Figure 6: Comparing the Blueline Tileifsh $F^{14}C$ (Blue) chronology (based on SCDNR age readings) to the A) South Atlantic Bight Wreckfish (Purple), the B) Gulf of Mexico Red Snapper (Green), C) Northwest Atlantic Halibut (Black), and Gulf of Alaska Pacific Ocean Perch (Red) reference chronologies. Error bars are defined as before. Shaded polygons represent 95% confidence intervals of $F^{14}C$ estimates based on generalized additive model smoothers.

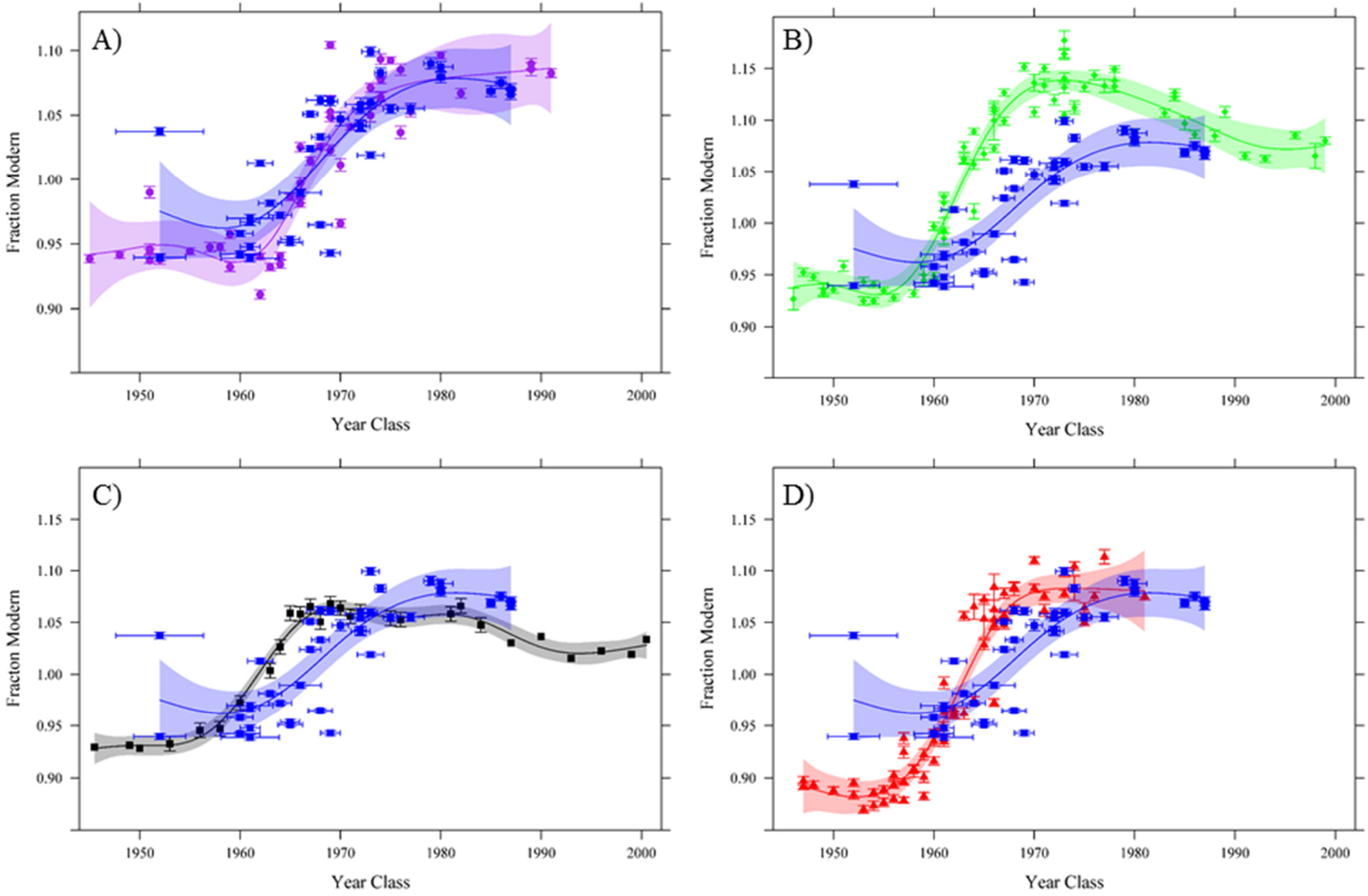
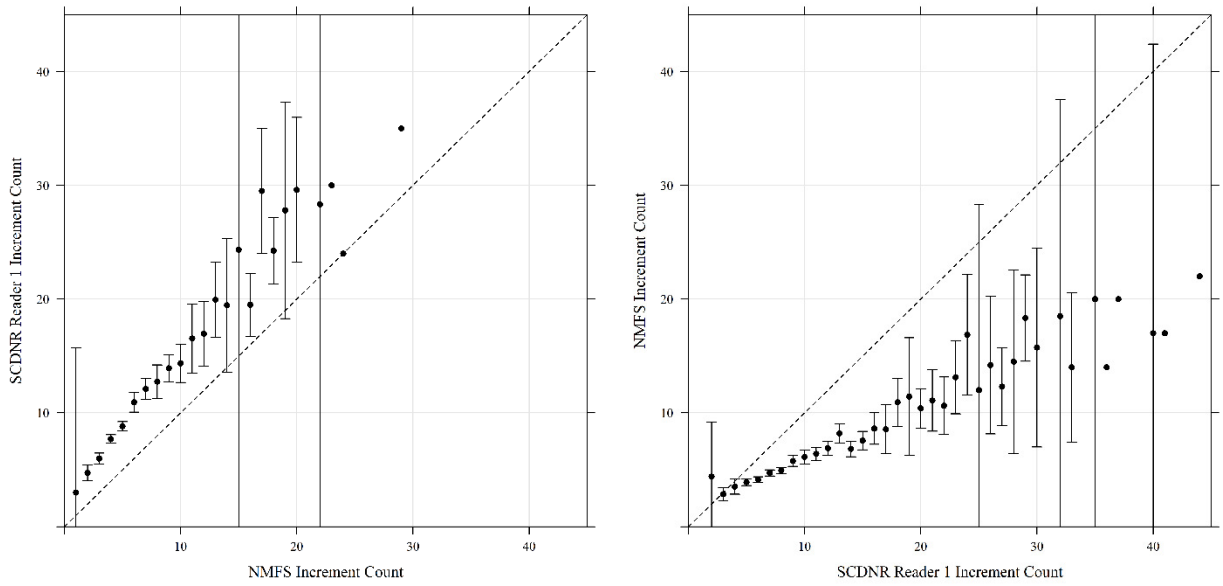
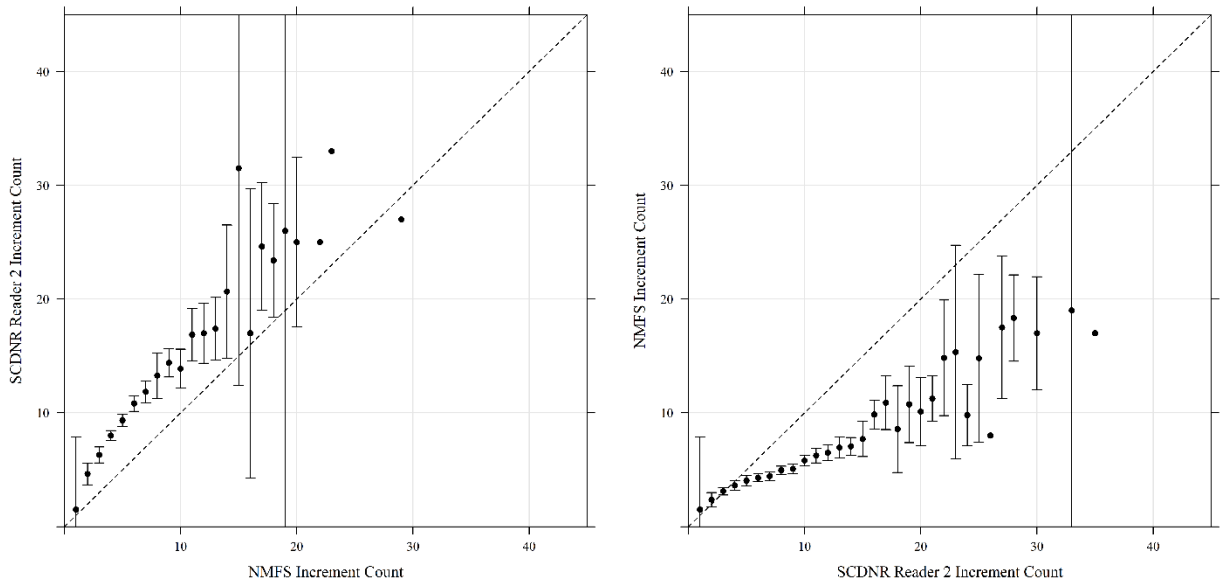


Figure 7. Results of paired readings of Blueline Tilefish from an exchange of 895 fishery-dependent samples collected by NMFS-Beaufort from 2013-2015 (see Table 7). The three panels represent, A) a comparison of SCDNR Reader 1 opaque zone counts to NMFS-Beaufort opaque zone counts, B) a comparison of SCDNR Reader 2 opaque zone counts to NMFS-Beaufort opaque zone counts, and C) a comparison of SCDNR Reader 1 opaque zone counts to SCDNR Reader 2 opaque zone counts. The dashed line is considered "true age" by reader on x-axis. The black dots are the average age reading compared to the "true age" by reader on y-axis. Error bars are 95% confidence intervals.

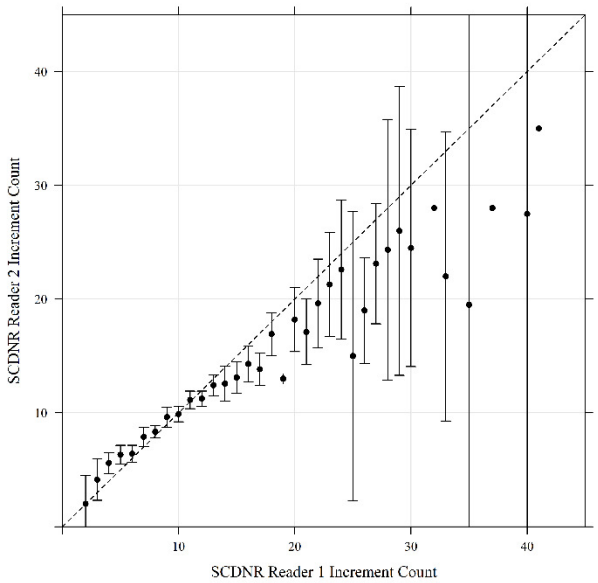
A)

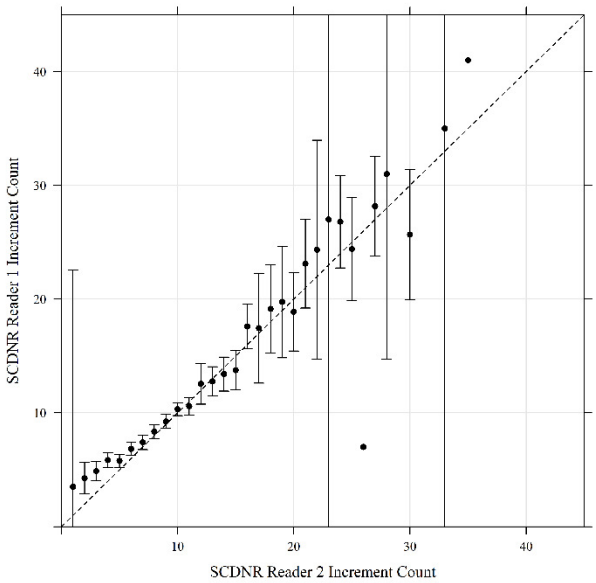


B)



c)


SCDNR Reader 2 Increment Count
SCDNR Reader 1 Increment Count
10
20
30
40


SCDNR Reader 1 Increment Count
SCDNR Reader 2 Increment Count
10
20
30
40

Appendix A.

Blueline Tilefish Aging Workshop

October 15 – 16, 2012

Beaufort, NC, USA

List of Participants:

Participant	Affiliation
Michael Cooper	National Marine Fisheries Service (NMFS)
Jessica Lewis	NMFS
Jennifer Potts	NMFS
Andy Ostrowski	NMFS
Joey Ballenger	South Carolina Dept. of Natural Resources (SCDNR)
Joe Evans	SCDNR
Tracey Smart	SCDNR
Betsy Laban	NOS/ SCDNR
James Davies	Old Dominion University (ODU)
Michael Schmidtke	ODU
Laura Lee	North Carolina Dept. of Marine Fisheries (NCDMF)
Stephanie McInerny	NCDMF

Processing Methodology

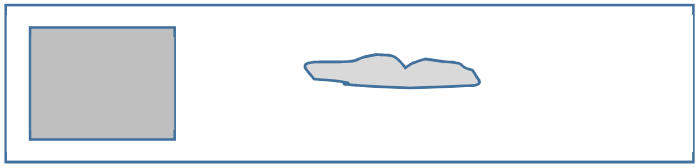
Processing methodology by the three labs (NMFS, SCDNR, and ODU) were similar, except the thickness of the final sections. The blueline tilefish otoliths have a deep sulcal groove, making the thin sections prone to breakage. To reduce breaking, all three labs embedded whole otoliths in epoxy resin. Differing from the other labs, NMFS staff took three serial transverse sections from the otolith encompassing the core using a single blade on a low speed saw to a thickness of 0.5 mm. The sections were mounted on slides using a thermo plastic adhesive and then polished using the grinding wheel on

the Hillquist to an average thickness of 0.32 mm. The final preparation was covered with a liquid cover slip.

Methodology used by ODU and SCDNR was similar in embedding and sectioning, but some materials and equipment varied; SCDNR and ODU used mounting medium that both adhered and covered sections on slides. No major differences in the appearance or readability of sections from NMFS and ODU were noted during the workshop, even though ODU sections were slightly thicker (0.35 - 0.40 vs. 0.32 mm). Older SCDNR sections were slightly thicker still than NMFS and ODU sections, making readability a potential issue.

Orientation of the sections on the slides differed between labs and was discussed. Preference of ODU and SCDNR were to mount the sections lengthwise on the slides, while NMFS was mounting sections crosswise (Figure 1). Discussion centered on readers tilting slides to better view the structure, which is easier when sections are lengthwise. NMFS will modify their process going forward to mount the sections lengthwise.

A.



B.

Figure 1. Orientation of sections on slides. A. ODU and SCDNR; B. NMFS.

Initial Age Comparisons

Prior to the workshop, calibration sets were exchanged between ODU and NMFS. Two readers from each lab blindly read both sets without prior knowledge of the others' readings. Readings were done on dissecting microscopes at magnifications no greater than 20 x using transmitted light. The overall average percent errors (APE) were calculated for each set and were approximately 15% among all four readers. Acceptable APE for difficult to read fish is 10%..

For both the NMFS and ODU calibration set, three of the six paired reader comparisons revealed no bias in counts between pairs. One reader, however, consistently read lower than the other readers for the NMFS set and higher for the ODU set.

Edge codes have been difficult to assign because of the diffuse nature of the opaque zones. Historical data available from MARMAP does not include edge codes, so data provided to SEDAR32 will assume opaque zone counts are equivalent to age. All age readings from this point further will include the edge type using the following criteria implemented by SCDNR/MARMAP (2002) and used in age studies of other reef fish:

1 Opaque zone on the otolith edge

2 Small translucent zone on otolith edge equivalent to <30% of the previous translucent zone

3 Moderate translucent zone on otolith edge equivalent to 30%-60% of the previous translucent zone

4 Wide translucent zone on otolith edge equivalent to >60% of the previous translucent zone.

Interpretation of Otolith Structure

As with many of the deep water species of fish, the structure of the otolith is difficult to determine. A section containing the core or very close to it is the preferred section to read due to ease in determining the first annulus and having a series of sections around the core area furthered interpretation. Overall, the annual increments appeared to be more easily distinguished in the lateral plane, rather than along the sulcal groove. Around the first eight opaque increments were best read as fields rather than distinct bands (Figure 2), while as the fish ages the increments become thinner and more uniform in width (Figure 3).

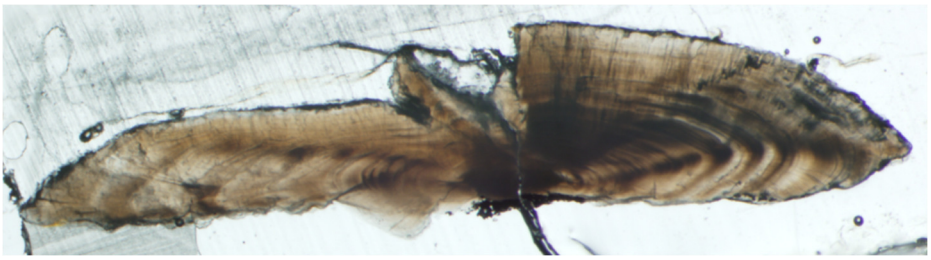


Figure 2. Blueline tilefish sagittal otolith section (8 year old). Otolith exhibits a translucent margin.

Figure 3. Blueline tilefish sagittal otolith section (17 year old). Older fish exhibiting how annuli become more defined and consistent in spacing after the first 6-8 annuli, which are more diffuse.

Aging Process Developed by Workshop:

1. Serial sections (2-3) encompassing the core should be taken from each otolith.
2. Finished sections should be between 0.30 mm and 0.40 mm thickness.
3. Use the section closest or with the core.
4. Identify an area that generally is clear to begin counting.
5. Define the 1st annulus. 1st annulus should be distinct from the core, separated by a translucent zone or field.
6. Begin counting increments.
7. Increments do not have to be distinct bands or lines. They are often fields, proximal to the core particularly for the first 6-8 increments.
8. An increment must be traceable around or at multiple places on the otolith. Not all will be discernible all the way around the otolith.
9. Some splitting does occur. Tracing will eliminate splits as separate increments.

Problems arise when:

1. The core section is not available
   a. The first annulus is difficult to define
   b. Increments are difficult to define

2. Sections are relatively thick or dense
   a. The translucent zone preceeding the 1st annulus is difficult to see and trace

   b. Separation of fields is difficult.

Follow-up Work

1. Define the first increment. Otolith measurements could assist with this, but will not be done prior to SEDAR32.

2. Marginal Increment analysis. Michael Schmidtke at ODU will be working on this as part of his Master's thesis.

3. Error matrix will be calculated for assessment model. Calibration sets from each lab will be exchanged and stratified to cover the size range (MARMAP Range of 300-1100 mm TL). Aim for 10 fish per size class with the following bins: < 350, 350 – 399, 400 – 449, 450 – 499, 500 – 549, 550 – 599, 600 – 649, 650 – 699, 700 – 799, and > 800 mm.

4. Historical calibration set from MARMAP will be read by the current age readers for blueline tilefish. Notes will be made on the quality of the section to include information such as whether the core section was available to read and if the thickness of the section impaired readability. These new age readings will be compared to "historic" reads to inform the data providers whether the MARMAP age data can be included with the current age data.

Appendix B

Standard Operating Procedure for Embedding and Sectioning Blueline Tilefish (*Caulolatilus microps*)

17 June 2016

Andy Ostrowski

NOAA Fisheries

Sustainable Fisheries Branch, Life History Group

Center for Coastal Fisheries and Habitat Research

101 Pivers Island Rd.

Beaufort, NC 28516

The purpose of this write-up is to describe the sectioning process of Blueline tilefish (BLT) sagittal otoliths.

Overview

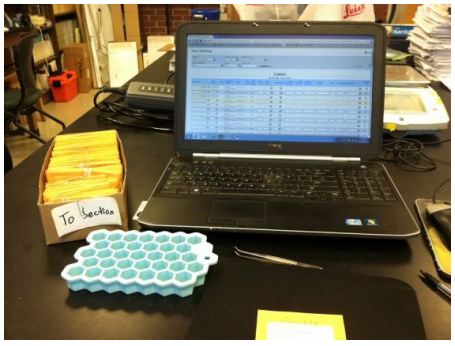
The best processing technique for BLT otoliths was embedding the sagittal otoliths in resin due to their frail nature from the deep sulcal groove. Prior to embedding in a two-part epoxy resin (Beuhler), the core area on each otolith was marked for ease when sectioning. Once the epoxy was hardened, excess epoxy material from the mold was trimmed off using the Hillquist machine or a dremel tool, and the remaining mold containing the otolith was affixed to a slide for sectioning. A set of three thin (~0.4-0.5 mm), serial sections was taken around the core of the otolith on a Isomet saw and then adhered on a slide in the order the sections were made. The middle section should contain the core. Once the sections were on the slide, they were polished on the Hillquist machine to a final section of 0.32 mm thickness. This further polishing after sectioning aids in clarifying annuli and the dense core of the BLT otolith for aging. A liquid cover slip, Gurr (aka DePex Mounting Medium), was applied to the sections and allowed to set up overnight before reading.

Preparation of embedding molds

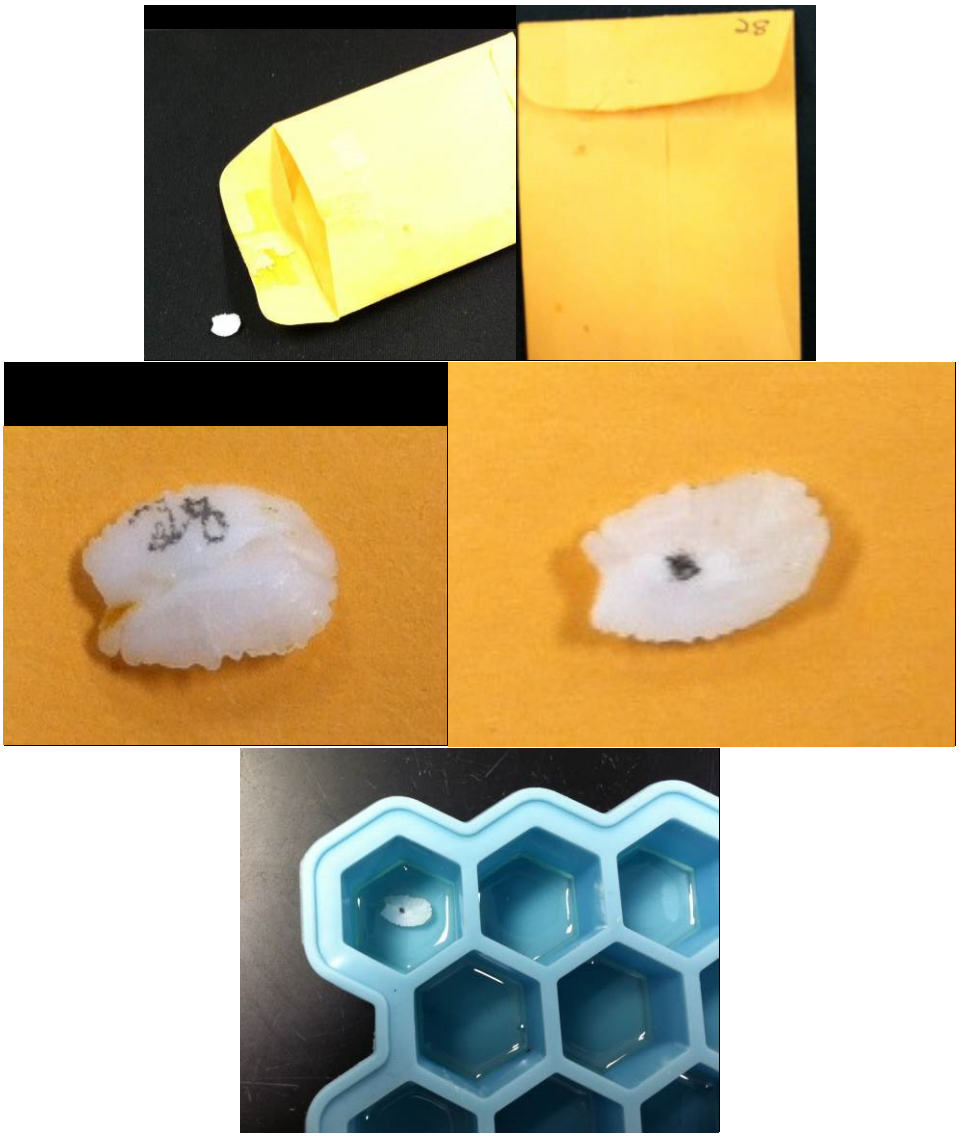
1. Prior to labeling samples, the bottom half of epoxy molds need to be poured and allowed to harden.
    a. See appendix for supplies, instructions and amounts.

Labeling samples

2. Put sample envelopes in order based on year and interview #.



3. Each slide box should contain 90 slides (due to label sheets), each with 1 sample per slide
4. Remove otolith sample from envelope, mark envelope and back of otolith with slide # (again 1-90), mark core area, and place into mold.

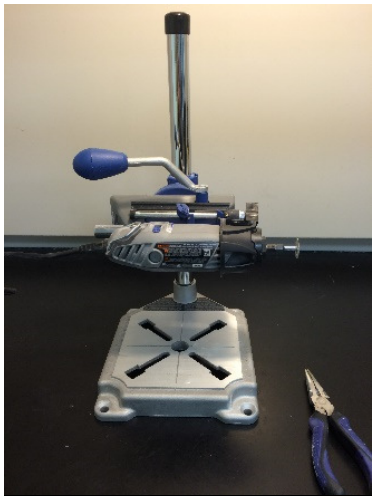


5. Once all samples have been labeled and otolith placed in half-poured, cured molds, finish molds by covering otolith samples with more epoxy as described in appendix and allow to dry overnight. Once cured, pop out all samples and prepare to trim on Hillquist.

Trimming

6. The excess hardened epoxy mold needs to be trimmed in order for the saw blade on the Isomet to cut completely through the sample. This can be completed on a Hillquist machine (or with the use of a Dremel rotary tool with diamond wheel attachment) and needle nose pliers. With pliers in hand, hold middle of sample, exposing one edge and apply to cutting wheel until complete. Switch sides of samples so that other side is exposed and repeat trimming process so that all that is left is a rectangular shaped mold. Repeat with until all samples have been trimmed.



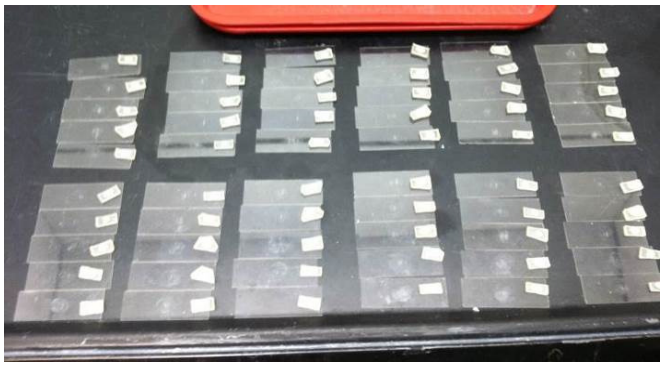



7. Put all samples in order from 1-90 on a tray. The sample number written on the proximal side of the otolith will be visible through the epoxy.

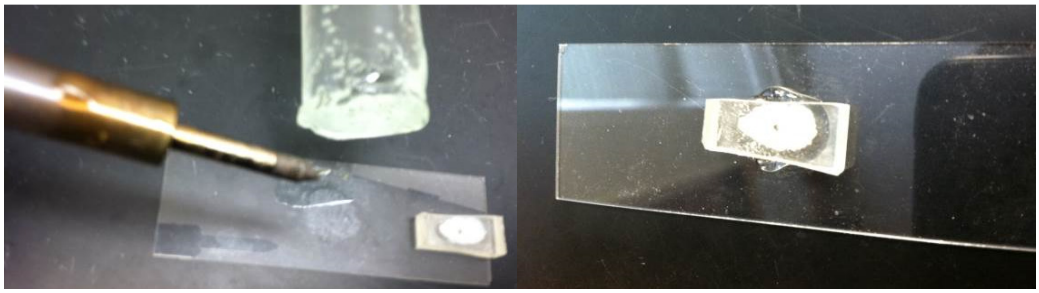
Sectioning

8. Supplies needed for fixing embedded samples to slides include: soldering iron, crystal bond (thermal glue), slides, and samples. Work needs to be completed in fume hood due to fumes when crystal bond is melted.

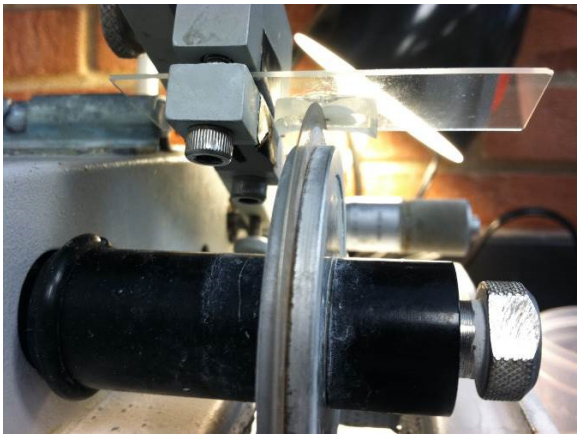
9. Line up all 90 slides and place samples on right edge of slide so that you can complete all samples in one sitting.



10. Once the soldering iron is hot, touch crystal bond to soldering tip over the middle of the slide and to left of sample. Allow 3-4 drips of crystal bond to drop on slide, lay crystal bond down, place embedded sample on liquid drops and push down, trying to line the otolith up parallel to bottom of slide. Be careful not to touch hot crystal bond as you will burn yourself. The crystal bond will cool rapidly, so placing and aligning the embedded otolith will need to be done quickly. Repeat for all samples



11. Line up all samples (IN ORDER) on tray for sectioning.



12. Sections are taken using an Isomet low-speed saw **at a speed of 9 and with the 75 and 25g weights (100 g total weight)**. **A single, 3 inch blade is used to section**. In order to ensure we have the core area and due to the difficulty in aging blueline tilefish, we take 3 serial sections. Methods for insuring consistency are as follows:

    a. Make sure the otolith is lined up correctly to get a true dorsal-ventral transverse cut of the otolith, adjusting the angle of the slide if necessary.

    b. With the saw off, line up the blade on the left side of the mark on the core area, laying it to rest on the blade.

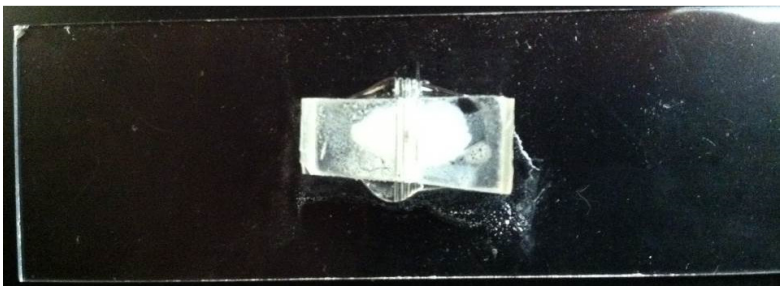
c. Noting which number on the saw micrometer arm, after lifting the sample off the blade, turn the micrometer knob one complete turn, twisting the knob counter clockwise, or toward you.

d. Lower the sample back on the blade, seeing if the turn has captured a majority of the marked core area. If it doesn't, adjust until comfortable with the two cuts.

e. Lifting the sample off the blade again, do one more rotation, again twisting the knob counterclockwise. This will be the location of the first cut, the furthest to the right.

f. Turn on the saw and lower the sample to begin cutting. Once the sample is finished cutting, (listen for the changes in pitch - it usually goes from low to high, indicating that the glass slide is being cut), lift the sample off of the blade, leaving the saw running, turn the knob one full rotation clockwise, or away from you.

g. Lower the sample, repeating the cutting process 3 more times until a total of 4 cuts are made on the sample. Remove sample from chuck. Sample should look like this.



13. Using forceps, remove samples from left to right and place on slide preloaded with dots of crystal bond in the same fashion, keeping sectioned samples in order.

14. Place slide on hot plate, allowing it to warm up before removing and, again using forceps, apply slight pressure on each section to "seat" the section and ensuring there are no air bubbles under the sample. Apply slide label to slide.

Polishing and Gurr

15. Again using the Hillquist, but this time on the grinding wheel side, polish the sections down to 0.32 mm, or 35 on the gauge.

16. After polished, each sectioned sample needs a liquid coverslip, Gurr, applied to it prior to reading. Line all samples on trays. Using a 45 degree tipped probe, apply Gurr by rubbing it on each section until all samples are completed. Allow to dry overnight.

Samples are now ready for age readings

Appendix B1: Mixing and Pouring molds

We found that the typical bullet molds were too small to hold some of the larger otoliths and would be too expensive to custom order. Instead, we used a silicon ice cube tray (http://goldas-inc.amazonwebstore.com/Outset-Ice-Cube-Tray-Hexagon-Small/M/B005A20RQ0.htm) and only filled with enough embedding material to cover the top of the otoliths. Any silicon style tray would work as long as the samples can easily be removed.

Notes:

A. Use Buehler EpoxiCure 2 Resin and Hardener at a ratio of 2.5g:1g, respectively.

B. Using the ice cube tray mold, 21 grams (total of both resin (15g) and hardener (6g) per mold is sufficient coverage for a bottom half. This works out to about 0.6g per individual mold.

    a. If embedding all three ice cube trays, a total of 63g: 45g resin and 18g hardener.

C. To cover the otoliths, 0.9 g per individual mold is enough. This works out to 22.5g resin and 9g hardener (31.5g total) per 34 samples

    a. If embedding all three ice cube trays, a total of 84g (due to 12 empty molds) with 60g Resin and 24g hardener.

Pouring molds:

1. Supplies needed: Silicon release spray, resin, hardener, trays, gloves, mixing cup, stirring rod, transfer pipette, paper towels, and scale. Make sure to complete in fume hood or well-ventilated area as process produces harmful vapors.

2. Spray ice cube trays with silicon release spray and wipe off excess spray. This process will extend the life of your trays.

3. Measure desired amount of resin then hardener into mixing cup (They can be mixed directly together, being very careful not to over pour, use of transfer pipette helped)

4. Mix well, stirring until there are no strings. Once bubbles are present and the mixture becomes clear, then the embedding medium is ready for transfer into the molds.

5. Once each mold has mixture in it, tip ice cube tray in all directions to ensure that the bottom is completely covered. Allow to dry overnight

6. Once otoliths have been placed in each mold, tops need to be completed (thus completely embedding otolith), repeat the process as above. Once mixture has been transferred, be sure to line up otoliths so they can be trimmed later. Allow to dry overnight.