Pseudokirchneriella subcapitata Chronic Toxicity Test Method

1. SCOPE AND APPLICATION

1.1. This method is based on EPA 821-R-02-013 *Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms*, 4th Edition, Test Method 1003.0. It is used to estimate the chronic toxicity of freshwater whole effluent and receiving waters on the, *Pseudokirchneriella subcapitata* (green alga*,* formerly known as *Selenastrum capricornutum*), using 4 to 7-day old organisms in a 96-hour static exposure. Tests covered by this procedure are BSY-CS-FSC and BSY-CD-FSC.

2. SUMMARY OF THE METHOD

2.1. Samples are received and prepared in the laboratory. Sample physical-chemical (phys-chem) parameters are measured and samples are adjusted as needed to meet permit or method test requirements. Sample dilutions and control waters are placed into test chambers. Test organisms are collected, and the inoculum is prepared. Test solutions are inoculated. Tests are incubated in laboratory incubators or on shaker racks during the duration of the test. Phys-chem parameters are measured and recorded daily. At 96-hours, aliquots from each test vessel are counted with an electronic particle counter. The test endpoint is growth (IC25). Statistical endpoints are obtained from the EPA WET Statistics Excel Spreadsheet per SOP TA-07.17. Results are entered into the Laboratory Information Management System.

2.2. Interferences: Refer to EPA-821-R-02-013 Section 14. Includes toxic substances introduced by contaminants in dilution water or on hands, glassware, sample hardware, and testing equipment. Dissolved oxygen <4.0 mg/L; pH <6.0 SU or >10.0 SU; salinity >1 part per thousand; ammonia, chlorine, and debris, indigenous organisms that may interfere with test organism.

2.3. Test acceptability criteria: Control minimum $1x10^6$ cells with EDTA. Control minimum $2x10^5$ without EDTA. No greater than 20% control variability.

3. EQUIPMENT AND SUPPLIES

3.1. Coulter Counter® model Multisizer 4e (SN SN BA23044), calibrated and used per SOP TA-06.41.

3.2. 250-mL glass Erlenmeyer flasks- autoclaved, 5 per test concentration

3.2.1. Five flasks per concentration plus five flasks for controls for each sample tested plus 30 flasks for a concurrent standard reference toxicant test. The flasks are alphanumerically labeled (e.g. A1) and are generally in sets of 21. **NOTE**: Four replicates are the minimum required by EPA method 1003.0—our fifth flask (each concentration) is used as a surrogate for parameters.

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3.2.2. 125-mL flasks can be used for the test; if used, reduce test volumes to 50 ml/replicate.

3.3. 1-ml and 10-ml individually wrapped, sterile, serological pipets with pump

3.3.1. One of each pipet per sample tested, plus one each for the control/dilution water, plus 8 for the PAAP nutrients.

3.4. 100-ml graduated cylinders, 6 per sample (for pouring up the test concentrations)

3.5. 1 L graduated cylinders, 2 per sample (for measuring test volumes)

3.6. 1 L volumetric flasks, 2 each (used to prepare the nutrient enriched sample)

3.7. Finnipipette 1000 µL pipettor and tips (used to transfer inoculum to test vessels)

3.8. Meters to measure:

3.8.1. pH (SOP TA-06.37)

3.8.2. Dissolved Oxygen (SOP TA-06.39)

3.8.3. Temperature

3.8.4. Conductivity (SOP TA-06.36)

3.9. Hach® kits to measure:

3.9.1. Total Residual Chlorine (SOP TA-06.22)

3.9.2. Alkalinity and Hardness (SOP TA-06.08)

3.9.3. Ammonia (SOP TA-06.35)

3.10. Rotating shaker platform

3.10.1. Used to prevent inoculum from settling to bottom of test vessels, reduce variability of light exposure, and facilitate air exchange in the test flasks.

3.11. Colored tape and a black-ink waterproof permanent marker

3.11.1. Used to label flasks and to identify glassware and plastic ware contents.

3.12. Magnetic stir plate

3.12.1. Used to mix samples after the addition of nutrients and to mix concentrations for definitive tests

3.13. 1'' to 2'' Magnetic stir bar

3.14. Magnetic stir bar retriever

3.15. Light meter

3.15.1. The light intensity of the incubator or tray where the test will be located is measured at test initiation and recorded on the bench sheets for each test. Refer to SOP TA- 06.26.

3.16. Computer with access to LIMS

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3.17. Specimen cups, 200 mL glass, 6 each

3.17.1. Used to hold samples of the control/diluent and the high, middle, and low sample concentrations for alkalinity, hardness, and conductivity measurements.

4. REAGENTS AND STANDARDS

4.1. Sample warmed to 25±1 °C

4.1.1. Two liters are required for chronic definitive; 500-mL for chronic screen. For a valid test: a) the sample should be held at 0-6°C prior to test initiation, and b) sample holding time should not exceed 36 hours from time of collection to test initiation. See EPA-821-R-02-013 for exceptions to above.

4.2. Test inoculum, *Pseudokirchneriella subcapitata* (culture 4–7 days old)

4.2.1. Prepare inoculum per SOP TA-07.11. Prepare inoculum no more than 2–3 hours before test initiation.

4.3. PAAP nutrients: (Prepared per SOP TA-09.09. Refer to EPA Method 1003.0.)

4.3.1. $MgCl_2.6H_2O$

4.3.2. $CaCl_2.2H_2O$

4.3.3. $NaNO_3$

4.3.4. $MgSO_4.7H_2O$

4.3.5. KH_2PO_4

4.3.6. $NaHCO_3$

4.3.7. Micro plus Trace

4.3.8. EDTA

4.4. PAAP media

4.4.1. Prepare per SOP TA-03.03. If metal toxicity is suspected, make PAAP media without EDTA.

4.5. Deionized (DI) water, autoclaved, with 500 mL wash bottle

5. SAMPLE COLLECTION, PRESERVATION, AND HANDLING

5.1. For a valid test: a) the sample should be held at 0-6° C prior to test initiation, and b) sample holding time should not exceed 36 hours from the time of collection (Time of last sample collection for grab samples or the end of the 24-hour collection period for composite samples) to test initiation.

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5.2. The remainder of each sample is stored in the Biology Walk-in cooler for the remainder of the test until it can be disposed of.

5.3. See SOP TA-06.14 for bioassay sample log-in procedure.

5.4. See SOP FS 1000 for sample collection and preservation.

6. TEST INITIATION

6.1. When the sample arrives, it is checked into the *Bioassay Sample Log-In* logbook and filtered but not autoclaved.

6.1.1. Log sample in per SOP TA-06.14. Filter per SOP TA-08.01.

6.2. Place clean 250-mL test flasks onto laboratory cart. Arrange the flasks in alphanumeric order (e.g., A1, A2, A3, A4, A5...). Identify the flask, the replicate (Control (CTL) A, CTL B, CTL C, CTL D, CTL E, etc.), the test concentration, and the facility/sample on the bench sheet(s). If the flasks are not identified alphanumerically or if one wishes to randomize the flasks, they can be labeled using the colored tape and waterproof marker.

6.2.1. Five flasks for each set of control replicates needed for each sample tested, plus five flasks per concentration for each sample tested, plus 30 flasks for the SRT if one is needed. (SRTs are performed either monthly or concurrently with the toxicity test). Mark the label with the concentration and the replicate (replicates A, B, C, D, and E). The "E" replicate is used to measure parameters during the test. Place a small piece of red tape on the "E" replicates as an aid in identifying the parameter flasks.

NOTE: Four replicates may be used per Method 1003.0. If four replicates are used, disregard references to replicate “E”.

6.3. Calibrate meters per respective SOP. Record calibration readings in the Lab Logbook section of the Laboratory Information Management System (LIMS).

6.4. Measure total residual chlorine (TRC) in sample per SOP TA-06.22. Record the result in the LIMS TOX_CHLORINE logbook and on the bioassay bench sheets. Check field data sheet supplied by sampler for field TRC and atypical field measurements. If TRC > 0.1 mg/L and non-chlorine toxicity is to be measured, dechlorinate a subsample (volume needed to initiate the test) of the sample using sodium thiosulfate per SOP TA-9.18. Setup a concurrent screening test using the 100% sample that has not been dechlorinated and a control. Record chlorine result, amount of sodium thiosulfate used and final chlorine result after dichlorination in the logbook and in the designated section in LIMS.

6.5. Fill a 30-ml disposable, plastic cup with sample and take initial pH measurement. If the pH is outside the acceptable range of 6.0-9.0 SU, then an additional test with a pH adjusted aliquot will be run. The test with the original sample is then analyzed as

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a screening test, regardless of whether the pH-adjusted aliquot is used for a screening or definitive test. Refer to SOP TA-08.06 for sample pH adjustment.

6.6. In same sample cup, take an initial DO measurement. If DO is ≤ 4.0 mg/L, aerate the entire sample at a rate of ~100 bubbles per minute to as near saturation as possible prior to test initiation. Samples with a potential DO problem generally show a downward trend in DO during the first few hours of the test. Monitor the DO closely, do not aerate samples in the test vessels. The rotating shaker rack facilitates air exchange in the flasks.

6.7. In same sample cup, take an initial conductivity, temperature and salinity (by direct measurement or conversion) of the sample. Record value in the Bioassay Sample Log-in Logbook and LIMS TOX_COND_SAL. Conductivity measurements can be rounded to the nearest 5 µmhos or 0.1 mmhos. If conductivity is greater than 2,000 µmhos or the salinity is greater than 1 ppt, saltwater organisms probably should be used rather than freshwater. The laboratory currently does not perform bioassay tests using saltwater algae. Check with the FDEP District personnel and check the Facility's NPDES permit for bioassay requirements.

6.7.1. If the sample is freshwater, the salinity will be too low to measure on the meter, use the following to convert conductivity to salinity: https://www.hamzasreef.com/Contents/Calculators/

6.8. Pour some sample into a 1-L volumetric flask, using a separate 1-mL pipet for each PAAP nutrient, add 1 mL of each PAAP nutrient to the flask containing the sample, then add sample to bring the flask to volume (the one-liter mark). Label the flask "sample plus nutrients."

6.8.1. If there is a known or suspected metal toxicity problem with the sample, the test should be set up with the PAAP nutrient EDTA omitted from both the sample and the control/diluent. Refer to comment #3.

6.9. For a screening test: Use a 100-mL graduated cylinder to pour 100 mL of PAAP (or PAAP-E) media into each of the control replicate flasks and a specimen cup. Measure 100-mL aliquots of the 100% "sample plus nutrients" into the 100% test concentration flasks and a specimen cup. Remove and replace foam plugs as needed.

6.9.1. Test volume size is 50 mL for a 125-mL flask and 100 mL for a 250-mL flask when an orbital shaker table is used for the test. Reduce the test volumes by one-half if the test is not to be continuously shaken. Five replicates for the control and each test concentration needed for each sample tested. Samples in the specimen cups (control and 100% for screening tests– control and all concentrations for definitive tests) are used for phys-chem parameters. Suggested sample volumes for a *chronic screening* toxicity test (for flasks only):

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Concentration	Diluent	Sample	Total Volume
Control	500 mL	0 mL	500 mL
l00 %	0 mL	500 mL	500 mL

6.10. **For a definitive test (0.5 dilution):** Measure the desired volumes of diluent and sample into separate labeled 500-mL graduated cylinders. Pour the contents of each cylinder into a labeled 1000-mL flask. Place the flask on the magnetic stir plate, place the stir bar into the flask, turn the stirrer on and let it mix. When the mixture is well mixed, remove the flask from the stirrer, and then remove the stir bar from the flask. Pour 100-mL of the solution into a 100-mL graduated cylinder, adjusting the final volume to 100 mL. Remove the foam plug from the appropriate test flask, pour the mixture into the flask, replace the foam plug, and then repeat for the replicates.

6.10.1. Five flasks per concentration in a dilution series for each sample tested plus extra volume for phys-chem parameters. Dilution series based on the permit and previous knowledge of toxicity level. An example of sample volumes when mixing a 1000-mL solution for a *chronic definitive* toxicity test with a 0.5 dilution factor

Concentration	Diluent	Sample/Test	Total Volume
Control	500 mL	0 mL	500 mL
6.25%	500 mL	500 mL of 12.5%	1000 mL
12.50%	500 mL	500 mL of 25%	1000 mL
25%	500 mL	500 mL of 50%	1000 mL
50%	500 mL	500 mL of 100%	1000 mL
l00 %	0 mL	500 mL	1000 mL

6.11. Perform QA Standard Reference Toxicant (SRT) tests monthly or concurrently if bioassays are performed less frequently. The SRT must be done 30 days prior to the start of a facility bioassay. FDEP Laboratory uses Cadmium Chloride as the standard toxicant. Other toxicants can be used.

6.11.1. Example of volumes and concentrations for *chronic definitive* cadmium chloride (concentration as cadmium) SRT test with extra volume for phys-chem parameters. Concentrations and toxicants can be modified as needed. Diluent is synonymous with control water.

Concentration	Diluent	SRT/Test	Total Volume
Control	600 mL	0 mL	600 mL
40 µg/L	600 mL	400 mL of 100 µg/L	1000 mL
60 µg/L	400 mL	600 mL of 100 µg/L	1000 mL
80 µg/L	200 mL	800 mL of 100 µg/L	1000 mL
100 µg/L	0 mL	600 mL of 100 µg/L	600 mL
120 µg/L	0 mL	1000 mL	1000 mL

6.12. Measure the temperature, DO, alkalinity, hardness, pH, and conductivity of the solutions in the specimen cups, and record the results in the appropriate spaces on the bench sheet. Record any comments pertaining to the condition or appearance of the sample.

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6.12.1. Conductivity readings shall be rounded to the nearest 5 µmhos. Readings for pH, DO, and temperature shall be read to one decimal place. Take measurements from lowest to highest concentration.

6.12.2. Record all readings in appropriate columns on *Chronic Bioassay Bench Sheet*.

6.13. Remove the foam plugs from the flasks and place 1 mL of inoculum into each test flask. Replace the foam plugs. Record the start time of this task.

6.13.1. Prepare inoculum per SOP TA-08.03 (10,000 cells/mL final in test solution)

6.13.2. The inoculation of the test solutions with the test algae marks the beginning of the test.

6.14. Place the flasks on a lab cart for transport to the incubator or shaker rack. Randomly remove the flasks from the cart and place the flasks in the incubator or on the shaker rack. Maintain continuous illumination of 400±40 foot-candles and a temperature of 25±1 °C for the duration of the test.

6.14.1. Random placement is used to reduce the possible influence on the test results of slight variations in illumination and temperature. If there is concern about temperature or illumination variations, the flasks can be rotated daily.

6.15. Measure and record (on the bench sheets) the light intensity at several places on the shaker rack, in the incubator, or on the culture tray where the test is placed. (Measure at liquid level).

6.16. Measure cell counts on three randomly selected flasks within two hours of test initiation.

6.16.1. Cell counts per SOP TA-06.41. This acts as a check on the cell density contained in the test concentrations (should be 10,000 ±10% cells per mL).

7. INTERIM READINGS

7.1. On days 1, 2, 3, and 4, (t = 24 hr, 48 hr, and 72 hr) perform cell counts on each flask (see comment). Record the cell counts and cell volume data on the *Chronic Bioassay Bench Sheet*. Thoroughly mix the test replicates by swirling each flask by hand if the flasks are not on a shaker rack.

7.1.1. All counting is done using the Coulter Counter® model Multisizer 4e electronic particle counter with mean cell volume attachment. Perform counts per SOP TA-06.41.

7.1.2. Toxic substances in the test solutions may degrade or volatilize rapidly so any inhibition in algal growth may be detectable only during the first day or two of the tests. **If this is not a concern, then counts need not be done until test termination at 96 hours**.

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7.2. On days 1, 2, and 3 (t = 24 hr, 48 hr, and 72 hr) measure the temperature and pH of the test solution in the "E" replicate flask of each test concentration (including the control). Record the measurements on the bench sheets.

7.2.1. Take physical-chemical readings carefully to minimize contamination and/or loss of test solution. If only four replicates are used in a test, measure Day 0 parameters in Flask “A”, measure 24 hours measure in Flask “B”, etc.

8. TEST TERMINATION

8.1. On termination day (t = 96 hr) perform cell counts on the solutions in each flask ("E" flasks do not need to be counted). Measure parameters of the solution in the "E" flask for each concentration (or “A” flask if “E” flask is not used). Record all counts and readings. Record any comments pertaining to the appearance or condition of the test solutions. Record test termination date and time.

8.1.1. Perform counts per SOP TA-06.41. Physical-chemical readings (pH, DO, and conductivity) are to be taken in "E" flask for each concentration. If only four replicates are tested, the 96-hour parameters are taken from Flask “A”.

8.1.2. Record counts, readings, date, and time in appropriate columns on *Chronic Bioassay Bench Sheet*.

8.2. Pour contents of all flasks into sink and flush drain with water. Place dirty flasks on cart for washing later.

8.2.1. Refer to Bureau Section Quality Manual for waste disposal.

8.3. Enter test results into statistical programs to obtain EC50, NOEC, LOEC and /or IC20 and IC25.

8.3.1. Per SOP TA-07.16.

8.4. Enter test results into LIMS.

8.4.1. Data is verified by a second person prior to being uploaded into LIMS.

9. DATA ARCHIVAL

9.1. Use a black, waterproof pen for all bench sheets and logbooks

9.2. To correct raw data entries, place a single line through the incorrect or illegible entry. Write the corrected entry near the error, date and initial the correction. Do not overwrite entries.

9.2.1. The corrected entry is written as near as possible to the error or in the comment box on the appropriate bench sheet.

9.2.2. If the correction requires an explanation, write a comment and reference the error with a letter. Initial and date the explanation. Do not attempt to put all the information (correction and comments) into a small space.

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9.3. Use sequential alphabetical annotation to identify errors and corresponding comments (use A for first error and use A for corresponding explanatory comment; use B for second error, etc.).

9.4. If a problem occurs during sample processing, a nonconformance report (NCR) is submitted describing the problem and the corrective action.

9.5. Record sample log-in in the Bioassay Sample Log-in book per SOP TA-06.14.

9.6. Record information in the *Pseudokirchneriella subcapitata Chronic Bioassay/SRT Bench Sheets* logbook and appropriate Laboratory logbooks in LIMS.

9.6.1. Log sample receipt, temperature, and condition into logbook. Fill in information requested at top of bench sheets as completely as possible prior to test initiation. Include full name of facility, address, contact person, date and time collected, field TRC, LIMS sample and job numbers, test chamber ID, test chamber size, and test solution volume. Access scanned field sheets via \\fldep1\dear\Labs\BizhubScans\Receiving

9.7. Enter the NOEC, LOEC AND IC25 results from the EPA WET Excel spreadsheet into the appropriate logbook, appropriate computer file and LIMS results editor. A second person must verify data prior to uploading into LIMS.

10. QUALITY CONTROL

10.1. Refer to FDEP Laboratory Quality Manual for corrective action and handling out-of-control data. Refer to EPA 821-R-02-013 Test Method 1003.0 for test conditions and acceptability criteria.

10.2. Perform QA standard reference toxicant (SRT) tests monthly or concurrently.

10.3. Samples should be held at 0-6 degrees C prior to test initiation (exception: hand delivered sample day of collection. Sample should be cooling relative to collection temperature). Sample holding time should not exceed 36 hours from time of collection until initial use of sample. Test temperature must not deviate more than 3° C in the test chambers for the duration of the test.

10.4. If a problem occurs during the sample processing or testing, the following steps are performed: 1) Lab supervisor is notified. 2) Client is notified (client makes decision for additional testing). 3) Section administrator is notified. 4) A non-conformance report (NCR) is submitted describing the problem and the corrective action.

10.4.1. To create a NCR: open LIMS and go to Laboratory/ Administrative/Non-Conformance Report. Refer to https://floridadep.gov/dear/quality-assurance/content/dep-sops for sample collection and preservation, and the Laboratory Quality Manual for corrective action and handling out-of-control data.

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11. SAFETY/HAZARDOUS WASTE MANAGEMENT

11.1. Proper laboratory protection, including lab hood or ventilation system, lab coat, gloves and safety glasses, is required when working with chemicals and unprocessed samples.

11.2. Refer to FDEP Laboratory Quality Manual for sample disposal.

11.3. Refer to the FDEP Laboratory Health and Safety Plan and Hazardous Waste Management.

11.4. Read MSDS information for reagent handling and disposal.

12. REFERENCES

12.1. EPA 821-R-02-013 *Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms*, 4th Edition, Test Method 1003.0.

12.2. DEP Laboratory Quality Manual Laboratory Quality Manual (state.fl.us)

12.3. DEP Laboratory Health and Safety Plan and Hazardous Waste Management health-safety-manual (floridadep.net)

12.4. DEP SOP TA-03.03, *Preparation of Preliminary Algal Assay Procedure (PAAP) Medium for Freshwater Algae Cultures*

12.5. DEP SOP TA-06.39 Thermo Scientific Orion Star A213 Benchtop RDO/DO Meter Calibration and DO Measurement

12.6. DEP SOP TA-06.08, *Measuring Total Alkalinity and Hardness*

12.7. DEP SOP TA-06.14, *Bioassay Sample Log-In Procedure*

12.8. DEP SOP TA-06.22, *Measuring Total Residual Chlorine using the Hach Pocket Colorimeter Test Kit*

12.9. DEP SOP TA-06.37 Calibration and Use of Fisherbrand FE150 pH Benchtop Meter

12.10. DEP SOP TA-06.36 Calibration and Use of the Fisherbrand Traceable Bench Conductivity Meter for Conductivity and Salinity

12.11. DEP SOP TA-06.41 Calibration and Use of the Coulter Counter Model Multisizer 4e

12.12. DEP SOP TA-07.16, *Using ToxCalc for Statistical Analysis of Chronic Bioassays*

12.13. DEP SOP TA-07.11, *Inoculation of Pseudokirchneriella subcapitata Toxicity Assay*

12.14. DEP SOP TA-08.03, *Inoculation of Algal Assay Tests*

12.15. DEP SOP TA.08.01, *Initial Algal Assay and Limiting Nutrient Sample Handling*

12.16. DEP SOP TA-08.06, *pH adjustment of Samples: Algal Assay and Toxicity Bioassay*

12.17. DEP SOP TA-09.18, *Dechlorination Procedure using Sodium Thiosulfate*

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12.18. DEP SOP TA-09.09, *Preparation of PAAP-Medium Stock Solutions*

12.19. DEP SOP TA-09.21, *Preparation and Testing of Cadmium Chloride and Copper Chloride Reference Toxicant Stock Solutions*

APPENDIX OF CHANGES:

08/28/2008	Moved *Scope and Application*, *Summary of the Method* and *Interferences* from comments to body of SOP. Added *References* and *Appendix of Changes*. Removed SOP TA-06.06 from Materials # 13 comment.
07/13/2009	Updated Quality Manual reference. Added "and IC25" to *Termination* Step 3.
08/14/2009	Replaced reference to SOP TA-07.08 with reference to SOP TA-07.16.
05/18/2010	Added 'CAUTION Individuals allergic to latex should not use latex gloves. A suitable substitute should be found and used.' and deleted 'Vinyl gloves can be substituted for individuals allergic to latex.' in Safety Section comments. Updated the reference to the Biology Quality Manual.
04/26/2011	Changed Biology Quality Manual reference to Bureau Quality Manual.
01/23/2014	Reformatted SOP.
06/12/2014	Corrected spelling errors.
02/12/2015	Formatted for 508 compliance.
06/16/2016	Updated links.
07/03/2017	Updated links. Clarified alkalinity and hardness measurements.
06/04/2018	Minor formatting corrections; changed font to Calibri.
05/31/2019	Updated Links and SOPs.
05/15/2020	Updated Links and SOPs. Changed and updated wording in most sections to reflect uniform language of SOPs
05/26/2021	Updated links

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