TITLE: Standard operating procedure for the identification and enumeration of potential toxin-producing phytoplankton species in samples collected from UK coastal waters using the Utermöhl method

Production Summary

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History of Procedure

Issue	Date Issued	Changes
1	11 April 2005	
2	08 June 2006	See version 1
3	26 July 2006	**Section 1: Introduction and scope** *Protoperidinium* species defined **Section 6: Environmental control** "a minimum" inserted and option to use 10 ml or 25 ml given **Section 8; Sampling and sample preparation** Time for settling of 10 ml subsample on sedimentation chamber defined **Section 9: Analytical procedure** Number of cells counted per field of view reduced from 10 to 4 Noted it may be necessary to conduct a count of the whole base of the chamber
4	March 2008	Grammatical changes made. Reordering of paragraphs. Distribution List modified Section 11 LOD calculation modified Section 13, Quality Control added
5	April - July 2012	Grammatical and formatting changes. Some wording clarified.

		Species list amended. Distribution list updated. New section 12, sample disposal and storage, added. Fields of View counting procedure (section 9.2) amended to ensure at least 100 cells of the target organisms are counted. Clarification and expansion of sections 14–16, quality control, acceptability criteria and corrective actions. Literature and reference section moved to end of the SOP. Figure 1 amended.
6	February 2021	Legislation updated *Phalacroma rotundatum* added, *Prorocentrum minimum* changed to *P.cordatum* and *Lingulodinium polyedrum* changed to *L.polyedra* in line with World register of Marine species nomenclature, New QC criterion for *Pseudo-nitzschia* spp. and *Prorocentrum cordatum* added Revised QC chart added Redundant websites removed

1. Introduction and Scope

1.1 The primary purpose of the toxin producing algae monitoring programme is to monitor UK coastal waters for the presence of those species of marine phytoplankton that are capable of producing biotoxins, in compliance with EU Implementing Regulation 2019/627 (UK retained legislation). Phytoplankton (microscopic floating plants) are an important food source for bivalve shellfish. Some phytoplankton species produce toxins and consumption of these species by bivalves may result in the accumulation of biotoxins in the tissue of the shellfish. If these shellfish are subsequently ingested by humans they may have a deleterious effect on human health.

1.2 The toxin producing algae monitoring programme forms part of the statutory shellfish flesh monitoring programme by providing an early warning of the presence of toxin producing species in UK waters and can be used as an investigative tool during the occurrence of shellfish toxin events. This SOP covers the identification and enumeration of those species and genera found in UK coastal waters that are capable of producing biotoxins. It should be noted that there are regional differences in the distribution and seasonal abundance of phytoplankton species in UK coastal waters including those able to produce biotoxins. There are also regional differences in the turbidity of the water and these differences vary according to weather conditions and hydrographic influences. For these reasons some regional variation in the counting methodology is necessary. Those species monitored throughout UK waters are listed below:

Alexandrium species (PSP)

Dinophysis species (DSP)

Phalacroma rotundatum

Prorocentrum lima (DSP)

Pseudo-nitzschia species (ASP)

Prorocentrum cordatum (Venerupin)

Lingulodinium polyedra (Yessotoxin)

Protoceratium reticulatum (Yessotoxin)

1.3 Whilst not part of this SOP, the occurrence of novel species, species occurring in high cell concentrations or those known as nuisance species (for example, *Karenia cf. mikimotoi* (associated with fish kills), *Noctiluca scintillans & Phaeocystis* spp.*)* will be noted so that where appropriate, additional investigative work can be decided.

2. Principle of the Method

2.1 A sub-sample of a preserved seawater sample is allowed to settle for sufficient time to allow phytoplankton cells to sink to the base plate of the sedimentation chamber (see sample preparation below). Either the whole sedimentation chamber or the base plate of larger volume chambers is placed on the mechanical stage of an inverted light microscope and the sub-sample is examined for the presence of specific, potentially toxin-producing or harmful species (see section 1.2 above). The volume of sub-sample settled, (50 ml, 25 ml, 10 ml or 5 ml), depends on the nature of each seawater sample. In general, 50 ml sub-samples are settled from coastal areas where there is little resuspended sediment or detrital material present, while 25ml (or smaller) sub-samples are settled from shallow or more estuarine sites. Where identification of cells is impaired by large amounts of sediment or detritus in the seawater sample, then a 10 ml or 5 ml sub-sample may be settled. It is important that sub-samples are settled on a firm, level surface. Phytoplankton species are identified by trained analysts using specified literature and references (including those which are hosted by various Internet sites). Counting of each of the target species or genus (see section 1.2 above) is undertaken and recorded by these analysts. Blooms of other species which occasionally dominate the phytoplankton community or the occurrence of so called 'nuisance species' are recorded but not always counted precisely.

2.2 The counting method used is determined by the density of different species present in the sedimentation chamber. The amount of sediment or detritus in a seawater sample will also influence the choice of counting method. If cells are present in low densities the entire bottom (base plate) of the chamber is scanned and all cells counted. This is the preferred method of cell enumeration. However, if cells are present at high densities (or if there is a significant amount of sediment or detritus present) then a number of fields of view (FOV) may be counted. Final cell concentrations are expressed as cells per litre (cells L^{-1}).

3. Reference Material

See section 18 for taxonomic reference texts.

4. Reagents

Detergent (5% Decon)
Distilled, deionized or Reverse Osmosis (RO) purified water

5. Equipment

Sedimentation chambers
Inverted microscopes with mechanical X-Y stage and vernier scale
Analysis recording sheet/laboratory book
Firm, level bench

Taxonomic keys/ literature (see list in section 18)
Calibrated stage micrometer
Micrometer eyepiece

6. Environmental Control

The sub-samples must be settled on a level bench and left without disturbance for a minimum of 4hrs (5 ml), 8hrs (10 ml), 12hrs (25 ml) or 20hr (50 ml). All analysis should be performed in an area free from vibration.

7 Interferences

7.1 Occasionally some of the water from the sub-sample may leak from the chamber if the seal between the glass base plate and metal base ring is broken. If there is any leakage from the chamber (evident as air bubbles at the top of the chamber) a further sub-sample is settled.

7.2 Some seawater samples may contain high cell concentrations, excessive amounts of detritus, or other material which may make species identification difficult and may cause some cells to be hidden. Where identification of cells is impaired by high cell concentrations, large amounts of sediment or other material then either 10 ml or 5 ml sub-samples of the original seawater sample should be settled and analysed.

8. Sampling and Sample Preparation

8.1 On arrival at the analytical laboratory, seawater samples should be logged in according to each laboratory's standard procedures and stored at room temperature until analysis has been completed.

8.2 The seawater sample bottle is gently inverted 10 times to ensure even mixing of the sample immediately prior to decanting a sub-sample into the sedimentation chamber.

8.3 The sedimentation chamber is filled to overflowing and covered with a cover slip making sure that no air bubbles are trapped.

8.4 The sample is allowed to settle in the chamber for the following period of time:

>4h 5 ml
>8h 10 ml
>12h 25 ml
>20h 50 ml

before analysis using light microscopy (See Section 2). The time of initial sedimentation should be recorded.

8.5 After each sample has been allowed to settle for the appropriate length of time, one of the procedures (as described below) is followed.

8.5.1 In Scotland and Northern Ireland a glass coverslip is slid across the base of the sedimentation tube enabling the sample tube and contents to be removed from the settled sample. The excess liquid is disposed of down the laboratory sink following local

COSHH guidelines. The base of the sedimentation chamber containing the settled sample is then placed on the microscope for viewing.

8.5.2 In England and Wales the whole sedimentation chamber (including the tube) is placed on the microscope for viewing.

9. Analytical Procedure

9.1 All enumeration should be performed using x20 objective and at least x10 eyepiece lenses. Higher magnifications may be required for species identification.

9.2 A quick scan of the settled sub-sample is performed to determine the method of enumeration to be used. One of two enumeration methods may be used, depending on the cell density of the target species or genus.

For statistical reasons it is necessary to count a minimum of 100 cells of the target species (if present). This can be achieved either by scanning the whole base of the chamber or by counting the cells in a number of fields of view (FOV). Scanning and counting cells across the whole base plate of the chamber is the preferred method of enumeration.

The second method is used when cells of the target species or genus are too numerous to ensure accurate counting over the whole base of the chamber. In these cases, fields of view (FOV) may be counted, the number of FOV being determined to ensure that a minimum of 100 cells of the target species are counted. If the FOV method is used, at least 10 FOV should be counted.

9.3 In most instances when the high density (FOV) method has been used it will still be necessary to scan the whole base of the chamber to count cells of less numerous taxa, since concentrations of different target species or genus may vary.

9.4 The taxonomic literature listed in Section 18, may be employed to aid identification.

9.5 As an aid to identification it may be necessary to manipulate cells using a needle to change their orientation in the chamber.

9.6 On completion of analysis the settled sub-sample is discarded according to the environmental, and health and safety procedures of each laboratory. The used sedimentation chamber is then left soaking overnight in detergent. The following day, the chambers are washed, rinsed with freshwater and wiped dry.

Low-density analysis (whole base plate).

9.7 Starting with the field of view at the top outer edge of the base plate, the mechanical microscope stage is used to move the base plate from left to right beneath the x20 objective lens of the microscope. When the field of view reaches the right hand edge of the base plate, a reference point, (e.g. cell or a piece of detritus) is identified and the microscope stage is moved down so that the new field of view is directly below the reference point. The microscope stage is then moved from right to left until the left edge of the base plate is seen in the field of view. Again a point of reference is taken and the stage moved to bring the field of view directly below that previously viewed.

9.8 This procedure is repeated until the whole of the bottom of the base plate has been viewed. Each cell of a potentially toxic species or genus that is observed, is identified, counted and recorded. If necessary, a clicker-counter may be used if cells are present in high numbers. Empty cells (whole or broken) are not included in the analysis. Where a cell can be identified and contains visible cell contents, whether a half cell or damaged, it should be included in the analysis.

9.9 Procedure 10.1 is then followed to calculate cell density.

High density analysis (fields of view).

9.10 Where cells of a target organism are present in high concentrations or it becomes impractical / time consuming to scan the whole base plate of the chamber, analysts may choose to either;

Settle a smaller volume of sub-sample and count the whole base of the chamber (as for low density analysis). Procedure 10.1 is then followed to calculate cell density, or;

Count the cells of target organisms in a minimum of 10 fields of view (FOV). The number of FOV being determined to ensure that a minimum of 100 cells of the target species are counted. (Procedure 10.2 is then followed to calculate cell density). In exceptional circumstances, it may be necessary to settle a smaller sub-sample volume and count fields of view.

10. Calculation of Results

10.1 Low density Analysis. For each individual target species, the concentration of cells/litre is calculated as;

$$\text{Number of cells observed} * \left(\frac{1000}{\text{volume settled (ml)}} \right)$$

10.2 High density Analysis. For each individual target species, the concentration of cells/litre is calculated as-

$$\left(\frac{\text{Total number of cells counted}}{(\text{Number of FOV})} \right) * (\text{Microscope field factor}) * \left(\frac{1000}{\text{volume settled (ml)}} \right)$$

10.3 Determining the microscope field factor annually or if the microscope is moved.

10.3.1 The diameters of the base plates of all phytoplankton settling chambers used for the identification and counting of potentially toxic phytoplankton species are determined using calibrated callipers.

10.3.2 For each sedimentation chamber, the internal diameter (mm) of the base plate should be recorded. Five replicate measurements of diameter should be taken for each base plate

and the mean calculated. The mean diameter for each chamber is then divided by two to obtain the mean radius of each chamber.

10.3.3 The mean radius of all the chambers used in this procedure is then obtained by summing the radii for all the chambers and dividing this number by the number of chambers measured. If sedimentation chambers are of different types or come from different batches then a mean radius must be calculated for each type of chamber and a note made of which chamber type was used for each sample analysed.

10.3.4 Using the formula for the area of a circle

Area = $\pi.r^2$
where **π** (Pi) = 3.1416
r² = Radius of the circle; squared.

the mean area of the settling chamber base plates can be determined. The mean radius of the base plates determined in 10.3.3 is used as the value for **r**. The results are expressed as mm^2.

10.3.5 Using a graduated microscope slide, the diameter of the fields of view at x200 magnification of each microscope used in this procedure is measured and recorded (mm). The radius of each field of view for each microscope is determined by dividing the diameter by two.

10.3.6 The area of a field of view at x200 for each microscope can be determined using the equation in 10.3.4 where

π = 3.1416
r² = Radius of a FOV; squared.

The results are expressed as mm^2.

10.3.7 Dividing the average area of phytoplankton sedimentation chambers (determined In 10.3.4) by the area of a FOV (determined in 10.3.6) gives the multiplication factor (known as the field factor) for each microscope at x200 magnification.

10.3.8 The sedimentation chamber number and its associated multiplication factor are then recorded on a Microscope Calibration recording sheet together with the date of calibration, magnification factor of the microscope used during the calibration (x200) and the microscope name/number.

10.3.9 The microscope multiplication factor (field factor) must be determined for each magnification of the microscope used for the identification and enumeration of potentially toxic phytoplankton.

11. Limit of Detection

The limit of detection of the method will be determined by the volume of the sub-sample used. The highest level of accuracy is achieved by using a 50 ml sub-sample and scanning the whole of the bottom of the base plate. Using this technique the Limit of Detection (in units of cells/litre = cells/L = cells L^{-1}) will be:

$$\left(\frac{1000}{\text{volume settled (ml)}}\right) \text{ cells / litre}$$

11.1 For analysis using FOV, the limit of detection will be:

$$\left(\frac{1}{\text{Number of FOV}}\right) * (\text{Microscope field factor}) * \left(\frac{1000}{\text{volume settled (ml)}}\right)$$

12. Sample disposal and storage

Following analysis each settled sub-sample is disposed of according to the environmental, and health and safety procedures of each laboratory. The remainder of the original seawater samples are retained for a minimum of 8 weeks.

13. Safety

See also COSHH/Risk Assessments for relevant SOPs.
A COSHH assessment must be produced by each analytical laboratory, for the handling and disposal of acidified or neutral Lugol's Iodine.

14. Quality Control

14.1 Internal Quality Assurance (QA) is conducted on a small percentage of the seawater samples collected. QA procedures are necessary to maintain confidence in the validity of results. The number of sub-samples used for QA purposes is dependent on the local Quality Control system in place at each laboratory and should be appropriate for the volume of work undertaken. Sub-samples that have already been analysed are selected for QA and are analysed by one or more different analysts independently and the results recorded as determined by the QA system of the laboratory (e.g. Internal QC form, electronic database). A minimum of 5% of samples should be selected annually for recounting. The results are compared, any discrepancies identified and corrective actions carried out as soon as possible.

Each QC identification and count must be performed on the same settled sub-sample as the original count.

14.2 It is recommended that the analytical laboratories undertaking this work take part in annual ring-trials (e.g. International Phytoplankton Intercomparison (IPI) exercise) to assess the performance of their analytical staff against similar staff in other Institutes. If performance is deemed unsatisfactory then some re-training may be necessary.

14.3 It is also recommended that the analytical laboratories undertaking this work should also seek formal accredited status such as that provided by UKAS 17025.

15. Criteria of Acceptability.

15.1 For those toxin producing species that have a trigger / action level above which action may be taken (Figure 1, path **a**):

15.1.1 If the result of the internal QC count is on the opposite side of the trigger/ action level to the original count, a second QC count is performed for the species in question. In addition, a non-conformance report is prepared and a corrective action is instigated (see 16 below).

15.1.2 If the first internal QC count produces a result that is on the same side of the trigger / action level as the original, the criteria defined in 15.2 apply.

15.2 For species which do not have a trigger / action level the following acceptability criteria is applied (Figure 1, path **b**):

15.2.1 For *Pseudo-nitzschia* spp. and *Prorocentrum cordatum* counts are deemed to be acceptable when both analysts count less than or equal to 1000 cells L^{-1} (ie 50 cells in a 50 ml sub-sample). If either analyst counts greater than this then the rules which apply to all other species will apply. That is, where both the original analyst and the QC analyst produce results fall within +/- 20% of each other, then the results are deemed to be acceptable.

15.2.2 If the original analyst and the QC analyst produce results which fall outside +/- 20% of each other, then the results are deemed to be unacceptable (except when criterion 15.2.3 applies).

15.2.3 Where either analyst counts 10 or fewer cells then a difference between analysts greater than 5 cells is deemed unacceptable. This applies to all species except *Pseudo-nitzschia* spp, and *Prorocentrum cordatum*.

15.2.4 If the result is deemed unacceptable under the criteria defined in sections 15.2.2 and 15.2.3 above, then a non-conformance report is prepared. A second QC count may be undertaken and a corrective action instigated (see 16 below) if deemed necessary by the laboratory technical manager.

16. Corrective action

16.1 For species with a trigger / action level, the results of the original count are compared to the first QC count (and second QC if performed) and a decision is made by the Technical / Quality Manager, in liaison with the analysts involved, on the agreed result for that sample. If this alters the outcome in comparison with the trigger level for that species, the following will take place:

16.1.1 A corrective action procedure is undertaken as defined by the local QC system to identify the reason for the discrepancy and develop a strategy to attempt to eliminate the problem and its recurrence (corrective action)

16.1.2 The revised result is reported to the necessary authorities following the reporting procedures as defined under the local QC system.

16.2 If the agreed result is on the same side of the trigger level as the original count, the criteria of acceptability as defined in section 15.2.1 above is applied.

16.3 For species without a trigger / action level the result of the QC count is compared to the original and a decision is made by the Technical / Quality Manager and analysts, as to the correct count for that sample. If the original result is not upheld the following will take place:

16.3.1 A corrective action **must be** undertaken to identify the root cause as defined above.

16.3.2 A decision is made by the Technical / Quality Manager whether to notify the necessary authority based on the revised result.

16.4 Persistent discrepancies in the results, or between two or more analysts, will result in a review of procedures and where necessary further training.

17. Uncertainty of Measurement

17.1 Sub-sampling

For this method, the preserved seawater sample is considered to be homogeneous once mixed.

The determination of the nominal volume of the settling chambers is considered sufficient for the accuracy required. The limits of acceptability for chamber volumes has been agreed by UK NRL analytical laboratories as +/- 2% of the nominal volume (see 17.2.3 below).

17.2 Instrument effects

17.2.1 Calibration of microscope

The graduated microscope slide and micrometer are purchased from reputable manufacturers working within required limits. (e.g. Accredited stage micrometers can be purchased from Agar Scientific Ltd, Essex).

17.2.2 Maintenance of microscope

The inverted microscopes are serviced annually by a certified engineer. Should a problem occur between services, the laboratory will request an engineer immediately and the microscope will not be used until the problem has been corrected.

The microscope eyepieces, lenses and filters are wiped clean with lens tissues before use.

17.2.3 Sedimentation chambers

Sedimentation chambers are purchased from reputable manufacturers working within the limits detailed below.

All chambers should have the same volume plus or minus 2%. The volume of each sedimentation chamber used in the procedure must be verified by weight annually.

All chambers should have the same base plate area plus or minus 1% as described by the measurement procedure in section 10.3.

Given the strict verification of the sedimentation chambers there is negligible contribution to uncertainty.

17.3 Storage conditions

The remaining original seawater samples should be retained for a minimum of 8 weeks following analysis (see section 12). It is preferential to keep the bottles in the dark and as cool as possible.

17.4 Analysis

Staff participating in the programme should complete in-house training prior to undertaking routine analysis of sub-samples.

Misidentification is minimised by ensuring that all staff are aware of the common dinoflagellate and diatom genera found in UK coastal waters. This reduces the risk of the incorrect reporting of potentially toxic species. Staff should be aware that in some instances discussion between several trained analysts may be required before a decision on identification is reached. This discussion is to be encouraged. However, phytoplankton identification can be difficult and it must be recognised that definitive species identification is not always possible.

18. Literature References

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Lindley, J. A. (ed.)(1993) "*Potentially Toxic Phytoplankton, 5. Genus Pseudo-nitzschia (*Diamtomophyceae/Bacillariophyceae)*",* ICES Identification Leaflets for Plankton No.185, 25pp.

Web sites

http://www.smhi.se/oceanografi/oce_info_data/plankton_checklist/ssshome.htm (Swedish Meteorological and Hydrological Institute)

http://planktonnet.awi.de (Alfred Wegener Institute for Polar and Marine Research)

https://www.io-warnemuende.de/gallery-of-baltic-microalgae.html (Leibniz Institute for Baltic Sea Research Warnemünde (IOW))

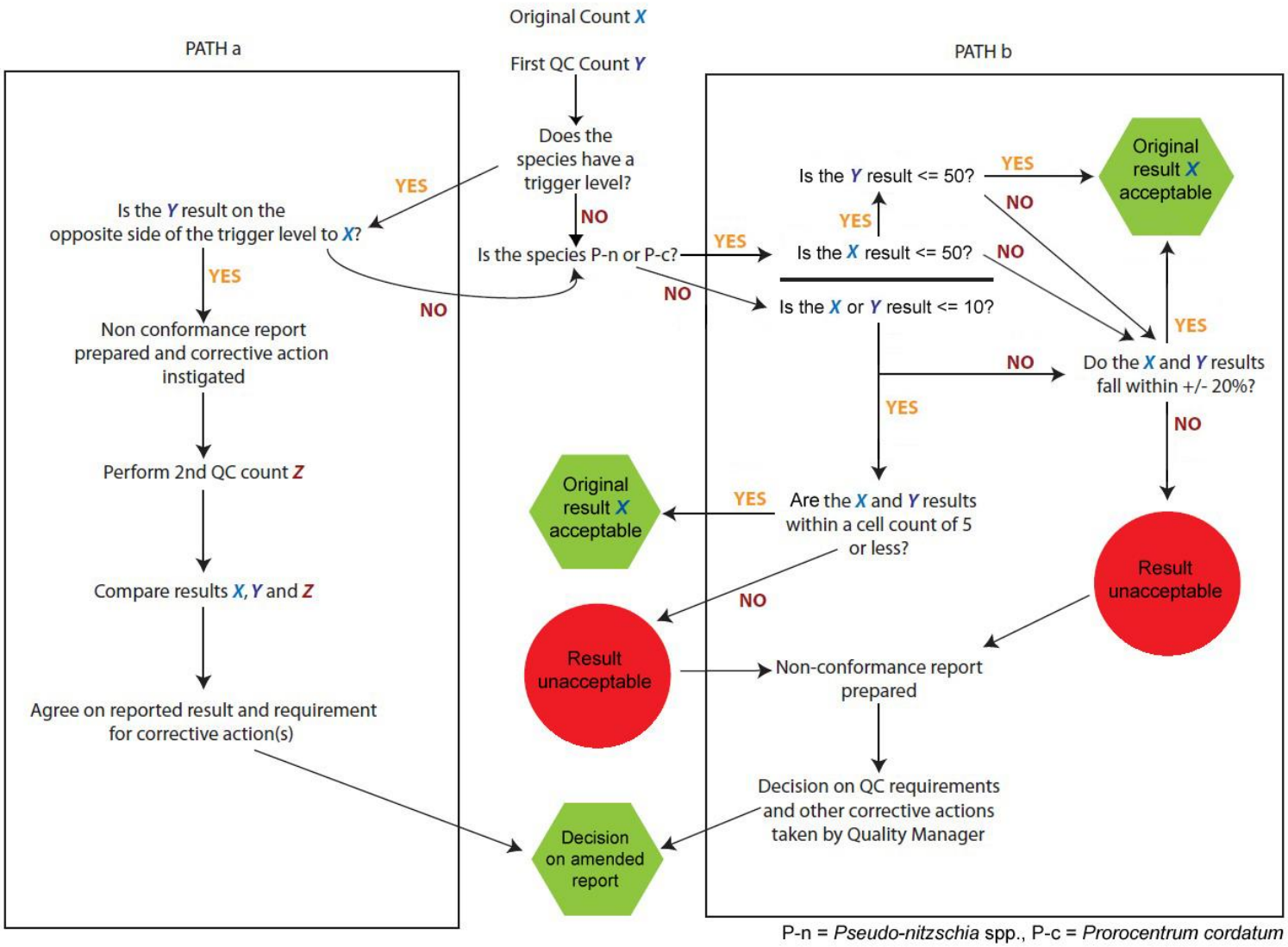

Figure 1. Diagrammatic representation of the processes describing the 'criteria of acceptability'.