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AQUAGAMETE

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Standardization of procedures and reporting in cryopreservation

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DEPARTMENT OF AQUACULTURE

Overview

- Why are we cryopreserving sperm?
- Identification of the problem
- Possible solutions to the problem
- Does standardization limit scientific freedom?

Why do we develop methods?

- To be used in aquaculture practice
  - to assist reproduction in aquaculture
  - to be applied to selective breeding programs
  - to be applied to conservation of genetic resources

Use of cryopreservation methods in aquaculture

- Very limited
- Applied on an individual basis
- Reasons:
  - sperm is seldom a limiting factor
  - limited use of selective breeding
  - lack of standardization

Standardization problems

Standardization problems

- Definitions
- Methods
- Publications

Typical problems

- Extenders
- Dilution ratios
- Cryoprotectant concentrations

Sperm Refrigeration and Cryopreservation

Fish milt was diluted 1:9 in an extender solution and stored at 4°C for 1 or 2 days. Four commonly used freezing solutions were selected in which high sperm survival has previously been reported: Steyn's medium containing 11% dimethyl sulphoxide (DMSO) (S1) or 9% glycerol (S4), and modified Mounib's medium containing 8% DMSO (M3) or 5% DMSO + 5% glycerol (M4) as cryoprotectants (21, 22, 29). A 10% fresh hen's egg yolk was added to both Mounib's media.

Fresh semen was gently mixed with the freezing solution at a ratio of 1:1 for Steyn's media (29) and 1:3 for modified Mounib's media (22). Aliquots of 300 µL of sperm/freezing media were pipetted into sterile 1.8 mL cryotubes (Nunc, USA), equilibrated for 5 min at room temperature and frozen using a two-phase freezing procedure in a computerized freezing unit (Orthodyne PTL P2D). In the first stage, they were cooled with liquid nitrogen (LN) vapor from room temperature to -5°C at 8°C/min. After a 4 min pause, cooling continued to -80°C at 8°C/min (29). Samples were subsequently stored in LN.

tion. Sperm were diluted 1:5 (sperm: extender) in a Kurokura-1 (16) extender (128.4 mM NaCl, 2.7 mM KCl, 1.4 mM $CaCl_2$, 2.4 mM $NaHCO_3$) and equilibrated for 40 min at 4°C. Ten percent pure dimethyl sulfoxide (Me_2SO) was added and every milliliter of mixture was transferred to a 2-ml cryotube, and then the cryotubes were directly transferred to a preprogrammed PLANER Kryo 10 series III at +4°C and cooled from +4 to −9°C at a rate of 4°C. min^{-1} and then from −11 to −80°C at a rate of 11°C min^{-1}, held for 6 min at −80°C, and finally transferred into liquid N_2. The spermatozoa were thawed in a water bath at 35°C for 110 s.

Problems with definitions

- Extenders
  - What is an extender?
- Cryoprotectant concentrations
  - In percentages or molars?
  - Relative to the final dilution or to the extender?
- Dilution ratios
  - Are they meaningful?
  - Sperm as a substance or sperm as a concentration of cells

Problems with methods

zero time (T_0). Sperm concentration was determined by counting cells in physiological solution at a final dilution of 1:1000 by means of a Burker cell haemocytometer under an Olympus microscope BX 41 (400×). The mean number of spermatozoa counted in 16 squares of the Burker cell was finally expressed as number of spermatozoa per unit volume.

The sperm dose used was 8.5×10^6 spermatozoa/egg with fresh sperm, and 17×10^6 with frozen one (the spermatozoa concentration was determined using a Neubauer chamber, three lectures were done per sample, and for each lecture, three different field of the chamber were recounted). Sperm was poured in a homogeneous way over the

Problems with methods

- Sperm concentration determination
  - Which method to use?
- Sperm cryopreservation methods
  - Equilibration
  - Handling of straws
  - Polystyrene box or controlled-rate freezer
- Calculation of fertilization and hatch rates

Problems with methods

The following motility parameters were chosen to evaluate sperm quality: rate of motile, locally motile, and immotile cells (%); averaged path velocity (VAP, μm s^{-1}); lateral head displacement (ALH, μm); and linearity (%) of motile sperm. Sperm were classified as motile sperm when the VAP was higher than 30 μm s^{-1} and locally motile sperm as sperm with a VAP from 10 to 30 μm s^{-1}. In the present paper, motile and locally motile cells are added up to "total motility" rates. Linearity is defined as straight-line velocity (VSL) divided by curve-linear velocity (VCL) as a percentage, which indicates that the higher the value is, the straighter or more linear the sperm path.

- Osmolality measurements
  - Wapor pressure or freezing point-depression osmometer?
- CASA measurements

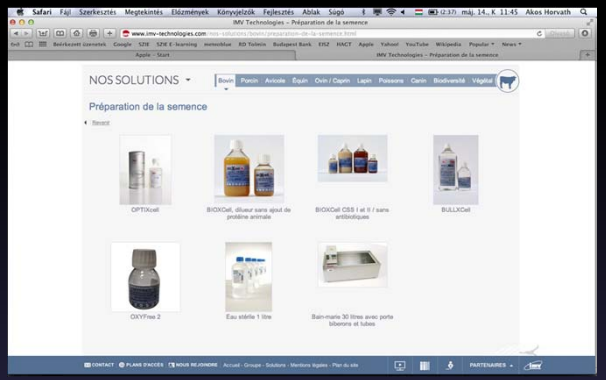
Problems with publication

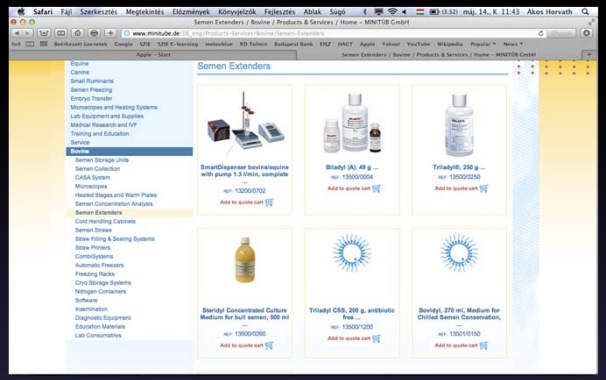
- Lack of guidelines or good laboratory practices
- Lack of standard procedures
- Lack of definitions

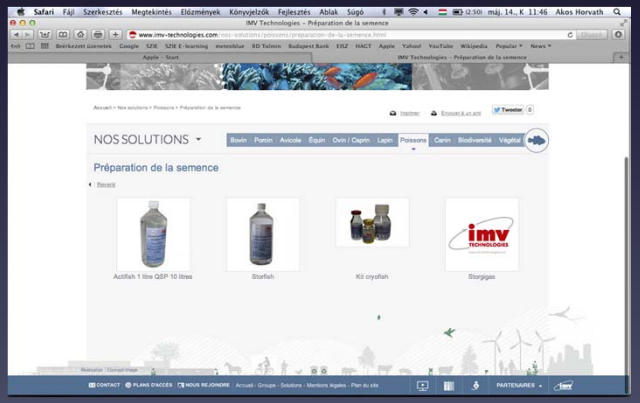
Problems with the industry

- Standards exist but are they applicable to aquaculture?
- Lack of branding
- Lack of quality control

Is there really a problem with the industry?







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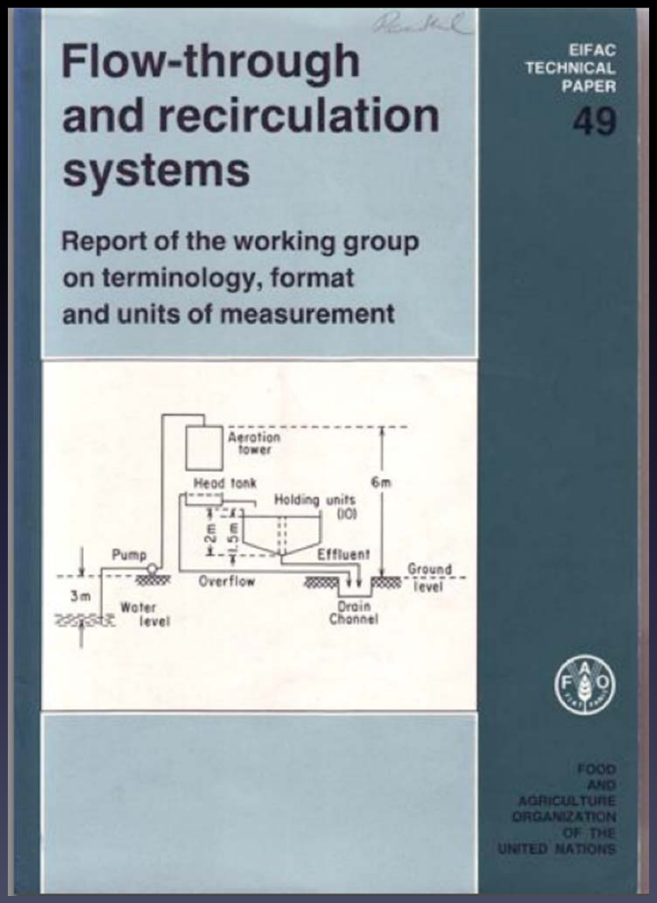
DEPARTMENT OF AQUACULTURE

Standardization

- Standardization of definitions
- Standardization of protocols
- Standardization of reporting

Standardization of definitions



1.1.8 Total alkalinity

The term "total alkalinity" refers to the total concentration of bases in water expressed in milligrams per liter of equivalent calcium carbonate.

Determination of alkalinity is carried out in two steps. First, by titrating with standard acid against phenolphthalein and second by titration against methyl orange indicator. The total amount of acid used in the titration, expressed as equivalent calcium carbonate, represents the total alkalinity. The amount of acid required to titrate to the phenolphthalein end point (pH 8.3), expressed as equivalent calcium carbonate, is the phenolphthalein alkalinity.

1.1.1.2 Total dissolved solids

The total residue remaining after evaporation of a water sample filtered to remove suspended matter larger than 1.0 µm (see particulate matter).

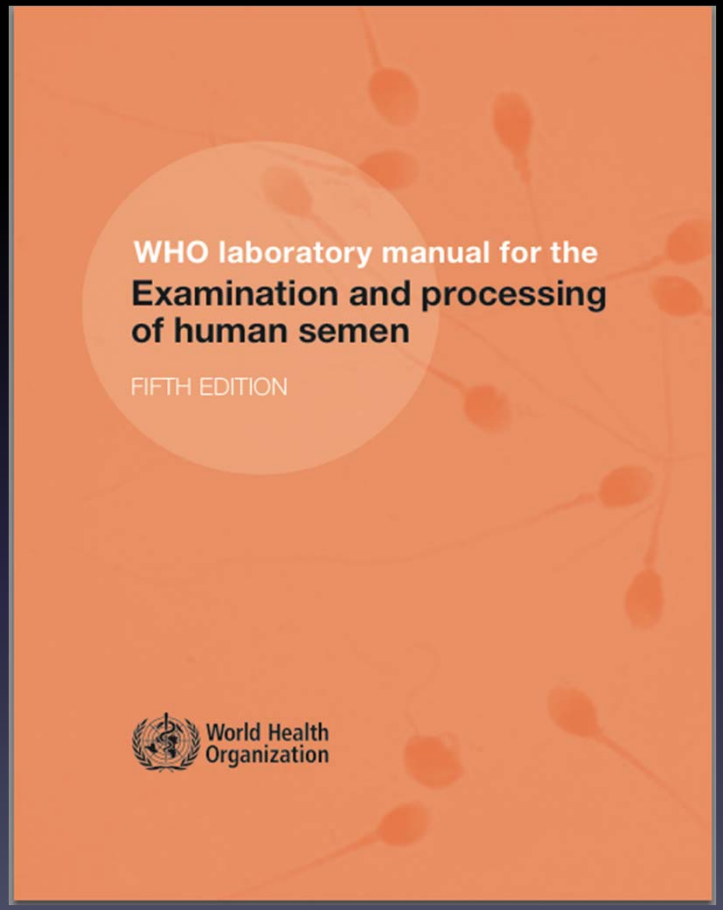
1.1.1.3 Dissolved volatile solids

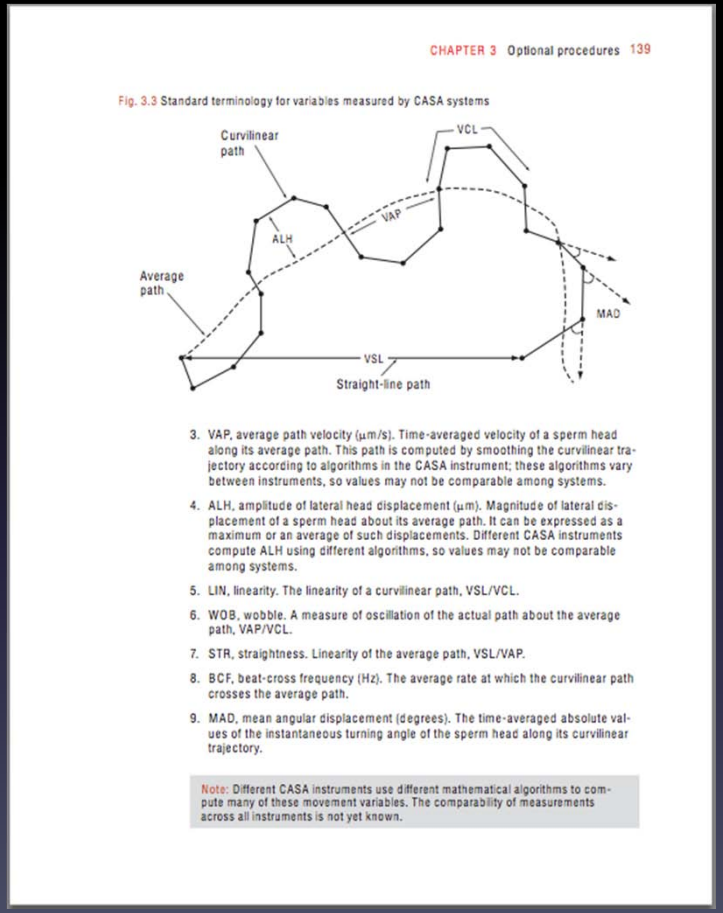
The residue from the total dissolved solid analysis is ignited at 550°C and the weight loss is determined. This measurement indicates the concentration of dissolved organic substances.

1.1.1.4 Total volatile solids

The quantity is the weight loss upon ignition at 550° C of the residue from the total solids analysis. However, the majority of investigators (at least outside the US) use ashing at 450°C. Total volatile solids are a measure of the dissolved and particulate organic matter.

Standardization of definitions





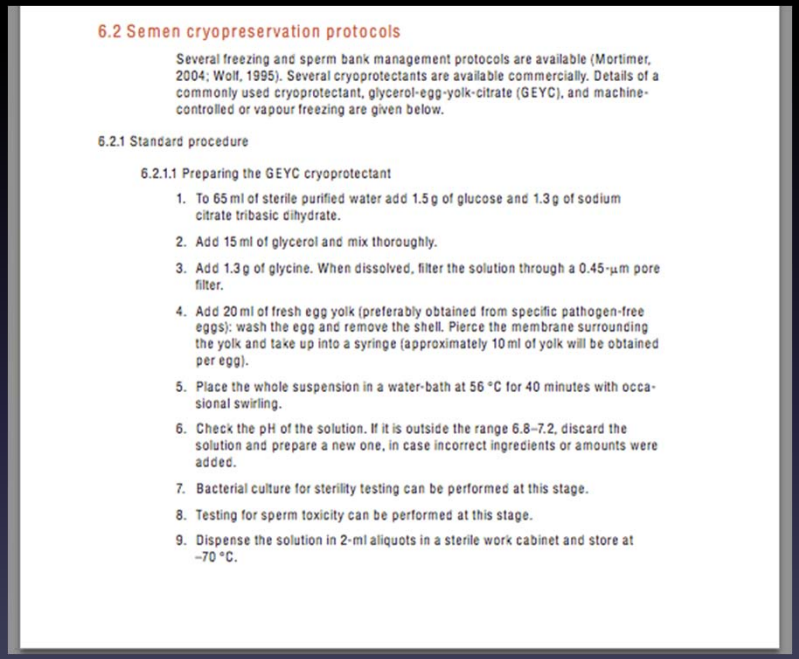
CHAPTER 3 Optional procedures 139

Fig. 3.3 Standard terminology for variables measured by CASA systems

3. VAP, average path velocity (μm/s). Time-averaged velocity of a sperm head along its average path. This path is computed by smoothing the curvilinear trajectory according to algorithms in the CASA instrument; these algorithms vary between instruments, so values may not be comparable among systems.
4. ALH, amplitude of lateral head displacement (μm). Magnitude of lateral displacement of a sperm head about its average path. It can be expressed as a maximum or an average of such displacements. Different CASA instruments compute ALH using different algorithms, so values may not be comparable among systems.
5. LIN, linearity. The linearity of a curvilinear path, VSL/VCL.
6. WOB, wobble. A measure of oscillation of the actual path about the average path, VAP/VCL.
7. STR, straightness. Linearity of the average path, VSL/VAP.
8. BCF, beat-cross frequency (Hz). The average rate at which the curvilinear path crosses the average path.
9. MAD, mean angular displacement (degrees). The time-averaged absolute values of the instantaneous turning angle of the sperm head along its curvilinear trajectory.

Note: Different CASA instruments use different mathematical algorithms to compute many of these movement variables. The comparability of measurements across all instruments is not yet known.

Standardization of protocols



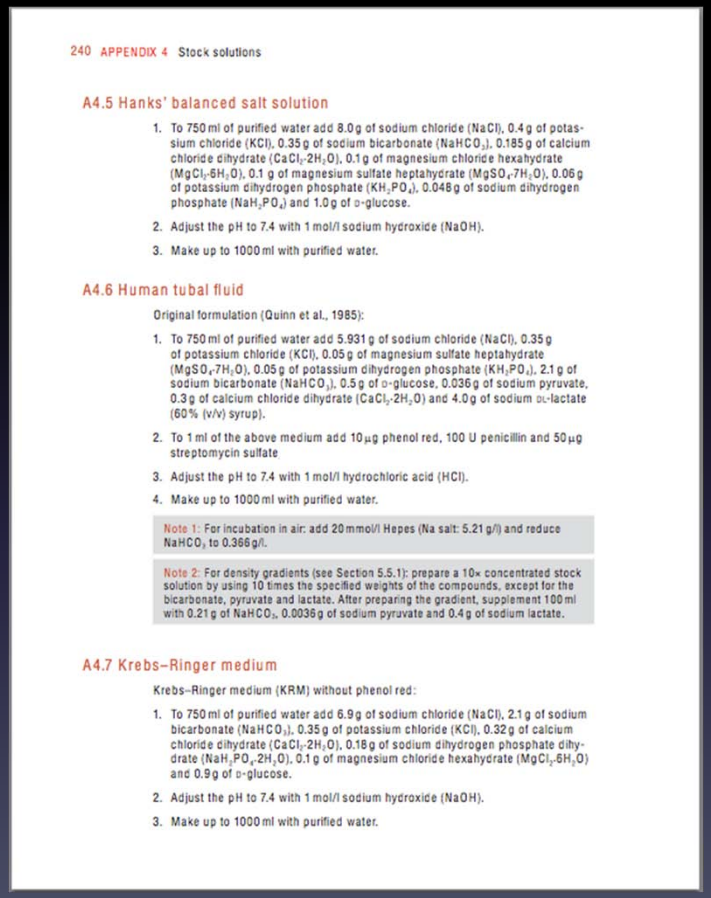
6.2 Semen cryopreservation protocols

Several freezing and sperm bank management protocols are available (Mortimer, 2004; Wolf, 1995). Several cryoprotectants are available commercially. Details of a commonly used cryoprotectant, glycerol-egg-yolk-citrate (GEYC), and machine-controlled or vapour freezing are given below.

6.2.1 Standard procedure

6.2.1.1 Preparing the GEYC cryoprotectant

1. To 65 ml of sterile purified water add 1.5 g of glucose and 1.3 g of sodium citrate tribasic dihydrate.
2. Add 15 ml of glycerol and mix thoroughly.
3. Add 1.3 g of glycine. When dissolved, filter the solution through a 0.45-μm pore filter.
4. Add 20 ml of fresh egg yolk (preferably obtained from specific pathogen-free eggs): wash the egg and remove the shell. Pierce the membrane surrounding the yolk and take up into a syringe (approximately 10 ml of yolk will be obtained per egg).
5. Place the whole suspension in a water-bath at 56 °C for 40 minutes with occasional swirling.
6. Check the pH of the solution. If it is outside the range 6.8–7.2, discard the solution and prepare a new one, in case incorrect ingredients or amounts were added.
7. Bacterial culture for sterility testing can be performed at this stage.
8. Testing for sperm toxicity can be performed at this stage.
9. Dispense the solution in 2-ml aliquots in a sterile work cabinet and store at –70 °C.



A4.5 Hanks' balanced salt solution

1. To 750 ml of purified water add 8.0 g of sodium chloride (NaCl), 0.4 g of potassium chloride (KCl), 0.35 g of sodium bicarbonate ($NaHCO_3$), 0.185 g of calcium chloride dihydrate ($CaCl_2{\cdot}2H_2O$), 0.1 g of magnesium chloride hexahydrate ($MgCl_2{\cdot}6H_2O$), 0.1 g of magnesium sulfate heptahydrate ($MgSO_4{\cdot}7H_2O$), 0.06 g of potassium dihydrogen phosphate (KH_2PO_4), 0.048 g of sodium dihydrogen phosphate (NaH_2PO_4) and 1.0 g of D-glucose.
2. Adjust the pH to 7.4 with 1 mol/l sodium hydroxide (NaOH).
3. Make up to 1000 ml with purified water.

A4.6 Human tubal fluid

Original formulation (Quinn et al., 1985):

1. To 750 ml of purified water add 5.931 g of sodium chloride (NaCl), 0.35 g of potassium chloride (KCl), 0.05 g of magnesium sulfate heptahydrate ($MgSO_4{\cdot}7H_2O$), 0.05 g of potassium dihydrogen phosphate (KH_2PO_4), 2.1 g of sodium bicarbonate ($NaHCO_3$), 0.5 g of D-glucose, 0.036 g of sodium pyruvate, 0.3 g of calcium chloride dihydrate ($CaCl_2{\cdot}2H_2O$) and 4.0 g of sodium DL-lactate (60% (v/v) syrup).
2. To 1 ml of the above medium add 10 μg phenol red, 100 U penicillin and 50 μg streptomycin sulfate.
3. Adjust the pH to 7.4 with 1 mol/l hydrochloric acid (HCl).
4. Make up to 1000 ml with purified water.

Note 1: For incubation in air: add 20 mmol/l Hepes (Na salt: 5.21 g/l) and reduce $NaHCO_3$ to 0.366 g/l.

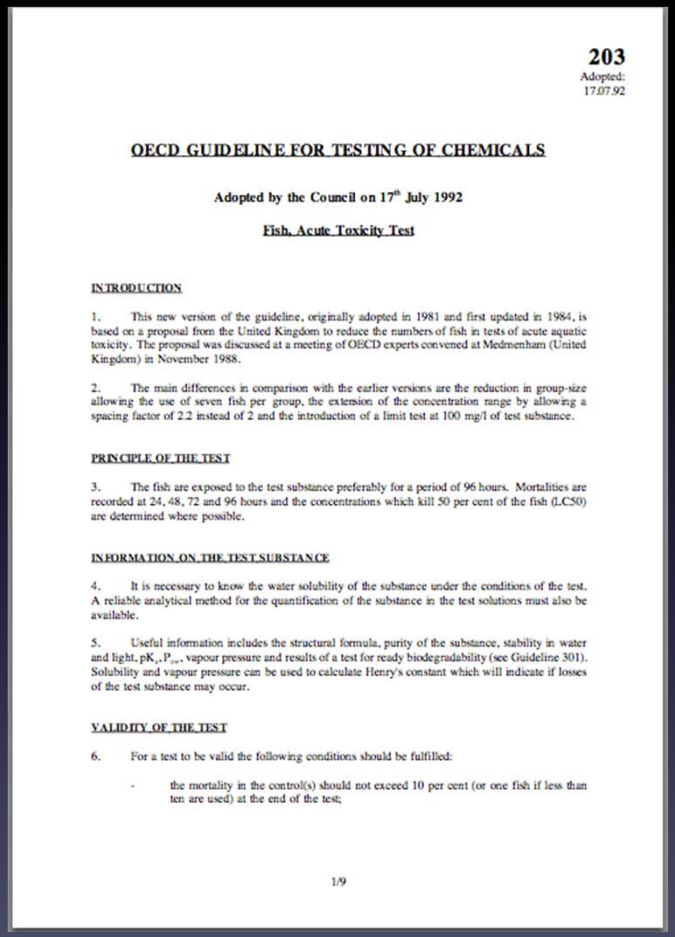
Note 2: For density gradients (see Section 5.5.1): prepare a 10× concentrated stock solution by using 10 times the specified weights of the compounds, except for the bicarbonate, pyruvate and lactate. After preparing the gradient, supplement 100 ml with 0.21 g of $NaHCO_3$, 0.0036 g of sodium pyruvate and 0.4 g of sodium lactate.

A4.7 Krebs–Ringer medium

Krebs–Ringer medium (KRM) without phenol red:

1. To 750 ml of purified water add 6.9 g of sodium chloride (NaCl), 2.1 g of sodium bicarbonate ($NaHCO_3$), 0.35 g of potassium chloride (KCl), 0.32 g of calcium chloride dihydrate ($CaCl_2{\cdot}2H_2O$), 0.18 g of sodium dihydrogen phosphate dihydrate ($NaH_2PO_4{\cdot}2H_2O$), 0.1 g of magnesium chloride hexahydrate ($MgCl_2{\cdot}6H_2O$) and 0.9 g of D-glucose.
2. Adjust the pH to 7.4 with 1 mol/l sodium hydroxide (NaOH).
3. Make up to 1000 ml with purified water.

Standardization of protocols



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Adopted:
17.07.92

OECD GUIDELINE FOR TESTING OF CHEMICALS

Adopted by the Council on 17th July 1992

Fish, Acute Toxicity Test

INTRODUCTION

1. This new version of the guideline, originally adopted in 1981 and first updated in 1984, is based on a proposal from the United Kingdom to reduce the numbers of fish in tests of acute aquatic toxicity. The proposal was discussed at a meeting of OECD experts convened at Medmenham (United Kingdom) in November 1988.

2. The main differences in comparison with the earlier versions are the reduction in group-size allowing the use of seven fish per group, the extension of the concentration range by allowing a spacing factor of 2.2 instead of 2 and the introduction of a limit test at 100 mg/l of test substance.

PRINCIPLE OF THE TEST

3. The fish are exposed to the test substance preferably for a period of 96 hours. Mortalities are recorded at 24, 48, 72 and 96 hours and the concentrations which kill 50 per cent of the fish (LC50) are determined where possible.

INFORMATION ON THE TEST SUBSTANCE

4. It is necessary to know the water solubility of the substance under the conditions of the test. A reliable analytical method for the quantification of the substance in the test solutions must also be available.

5. Useful information includes the structural formula, purity of the substance, stability in water and light, pK_a, P_{ow}, vapour pressure and results of a test for ready biodegradability (see Guideline 301). Solubility and vapour pressure can be used to calculate Henry's constant which will indicate if losses of the test substance may occur.

VALIDITY OF THE TEST

6. For a test to be valid the following conditions should be fulfilled:

- the mortality in the control(s) should not exceed 10 per cent (or one fish if less than ten are used) at the end of the test;

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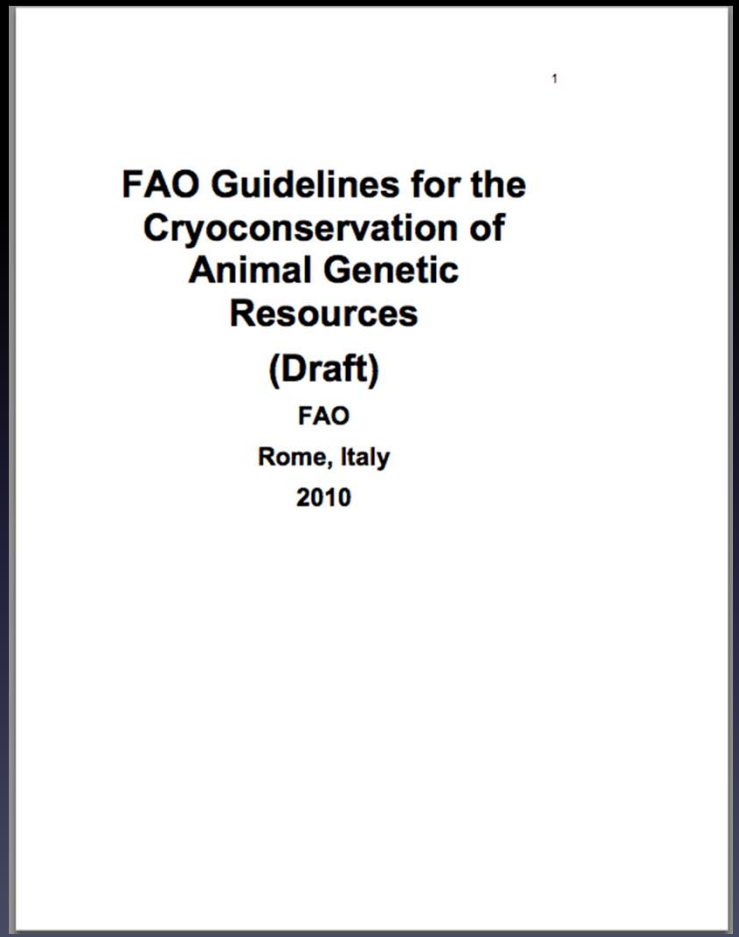

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FAO Guidelines for the Cryoconservation of Animal Genetic Resources

(Draft)

FAO

Rome, Italy

2010

Standardization of reporting

RECOMMENDED GUIDELINES FOR THE DESCRIPTION OF CULTURE SYSTEM OPERATION

1. Total Systems

1.1 Total water volume of system

Describe whether or not this figure includes water masses contained in pipes, sumps, reservoirs, pumps, etc. Water flow rates and/or retention times should be provided. Indicate whether total flow is identical with the flow rate through the culture unit; flow rates through bypass-systems must be reported.

1.2 Percentage recycling

The extensively used term "percentage recycling" is applied variously in the literature without providing sufficient interpretation. For example, in most of the North American literature "90 percent recycling" means that 10 percent of the flow is exchanged during each turnover, regardless of the time required for each cycle (Appendix 2, Figure 1a). Frequently the term "10-pass-system" is used to describe operational conditions. Unfortunately, the time required to complete a full cycle is seldom indicated. In most of the European literature, the term "90 percent recycling" generally indicates that 10 percent of the water mass contained in the total system is exchanged per day, regardless of the number of cycles during this time period (Appendix 2, Figure 1b). The Working Group recommends that the term "percentage recycling" always be defined clearly in reference to the operation being described. It is also considered essential to indicate the time required for the total water volume to complete the cycle. If multiple loop systems are employed, it is of interest to report not only calculated turnover times from pump capacity but also flow rates to system components.

Example: standardization for high-throughput cryopreservation of hybrid catfish (E Hu, High-throughput sperm cryopreservation of aquatic species, PhD Dissertation, Louisiana State University, 2012)

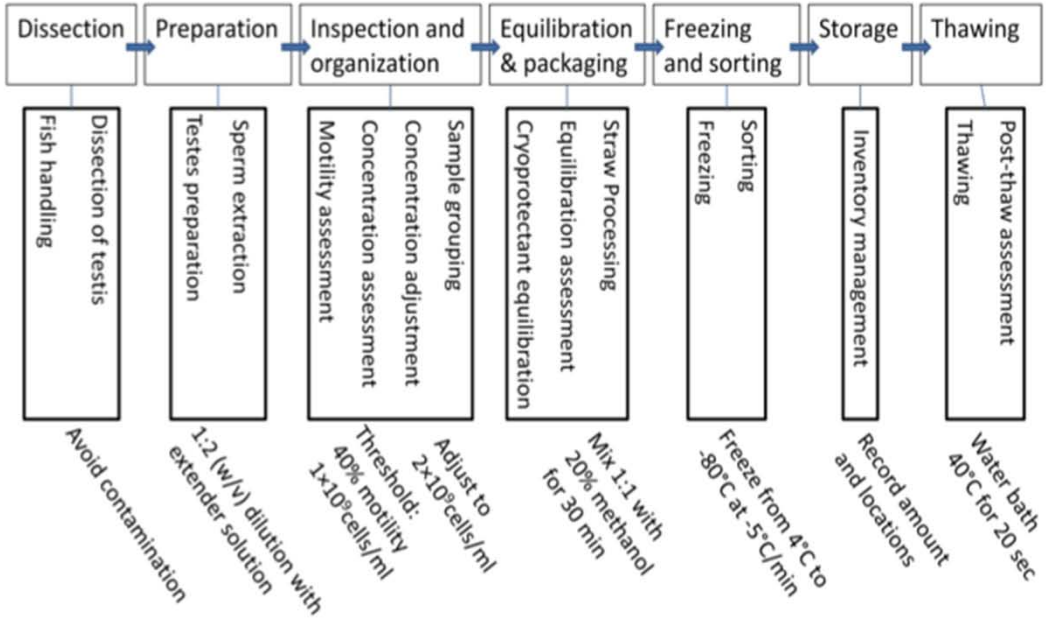

Figure 8.2. Schematic overview of cryopreservation of catfish sperm using automated processing. The central process elements were divided into seven steps (top, connected by arrows), each step entailed specific activities (upright rectangles) that were optimized individually by research experiments. The optimized steps were integrated into standardized protocols (specific examples provided at bottom in angled text).

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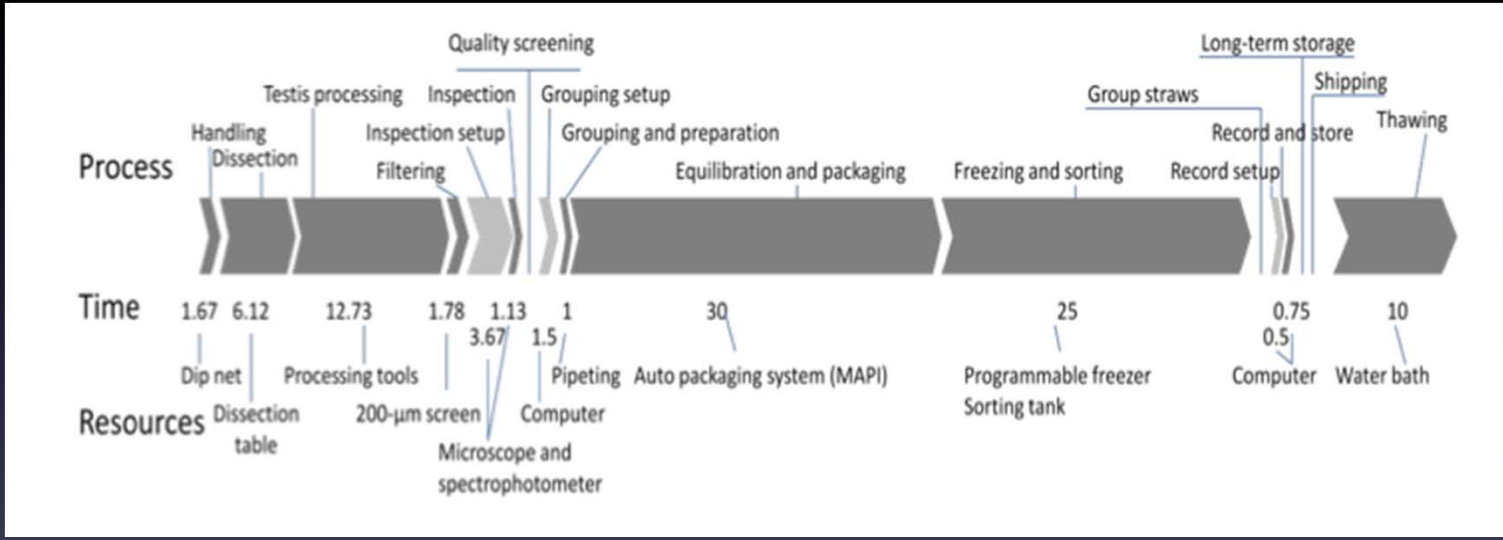

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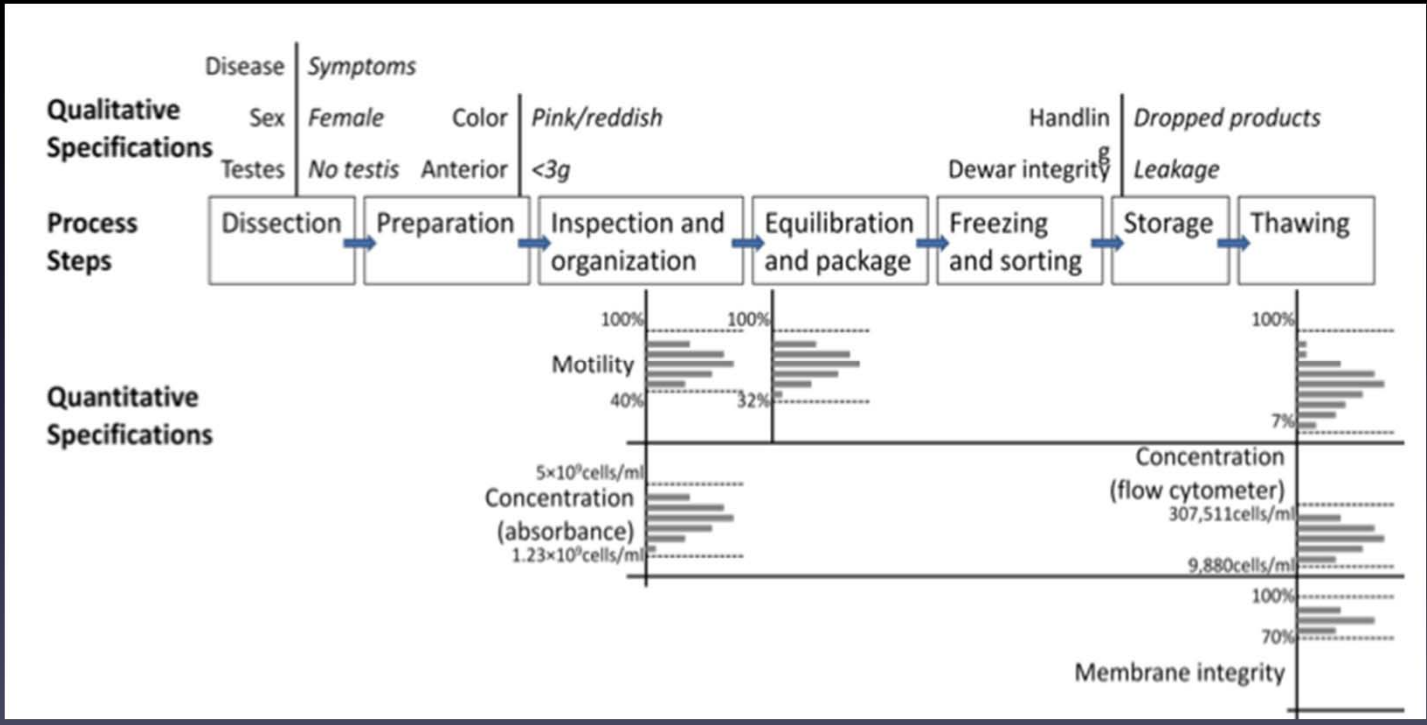

Acceptance by scientists

- Does it limit scientific freedom?
- Do I have to follow the published protocols?

Acceptance by scientists

- Scientific freedom ⟷ sloppy reporting
- No application without standardization
- Standard terms can be used as a reference and a starting point