Skimmed milk flocculation SOP for irrigation water

The aim of the protocol is to concentrate viruses present in 10L of water into a final volume of 10 mL (5 mL in clean water to test higher volumes) of phosphate buffer.

Equipment:

10L plastic containers with flat bottoms	☐
High speed centrifuge (8,000xg)	☐
Ph-meter and calibration solutions	☐
Sterile graduated disposable pipettes	☐
Sterile plastic tubes of 1.5 and 10-15 mL	☐
Centrifuge pots (500 mL)	☐
Magnetic stirrers and magnets (one per sample)	☐
A peristaltic pump for removing the supernatant (optional)	☐
Timer to switch-off the stirring after 8-10 hours	☐
Electric strip	☐

Reagents (see appendix):

Hydrochloric acid (1N and 0,1N)	☐
Sodium hydroxide (4% w/v = 1 M)	☐
Skimmed milk powder (Difco Ref. 232100)	☐
Phosphate buffer (1:2 v/v of sterile Na_2HPO_4 0,2M and NaH_2PO_4 0,2M at pH 7.5)	☐
Artificial sea salts (Sigma Ref. S9883)	☐
MS2 phage (10^7GC/ml per sample)	☐
Sodium thiosulphate (10%)	☐

Preparation of Pre-flocculated Skimmed Milk (PSM):

Check the calibration of the pH-meter in the alkaline and acidic range and recalibrate if necessary. Always disinfect the pH-meter electrode with fresh hypochlorite solution (0,1N). Prepare 100 mL of PSM 1% (w/v) for each water sample JUST BEFORE TO BE USED. In a litre of artificial seawater (33,33gr artificial sea salts into 1L of sterile H_2O) add 10gr of skimmed milk powder. Stir with a magnetic stirrer and AFTER DISSOLVING adjust the pH at 3.5 (± 0.1). DO NOT AUTOCLAVE.

Conditioning of water and flocculation:

10 litre water samples must be preconditioned before flocculation. If samples present high quantity of suspended material (sand or algae) let it sediment for 15 minutes and poor carefully the water into a new container. If samples present free chlorine, add 100ml of a 10%

thiosulphate solution (previously autoclaved) and let it stir for 10 minutes. Use one different plastic pipette for each sample.

Add a magnet into the bottle and adjust conductivity by the addition of 10gr of artificial sea salts into each sample. AFTER DISSOLVING adjust the pH at 3.5 (± 0.1) by the addition of HCl 1N. Mix the water thoroughly by vigorous stirring while adding the HCl. This step is important for the concentration of the viruses so make sure the pH has been properly adjusted. Use a new pipette in each sample. Add 100 mL of PSM 1% to each water sample.

Stir the samples for 8 hours to allow the viruses to adsorb to the flocks. Use a timer for automatically switch off the stirring and let the flock's sediment by gravity for 8 hours.

Negative process control:

A negative process control should be included in every batch of concentrated samples. Use 10L of tap water containing 100 ml of a solution of 10% sodium thiosulphate to eliminate residual free chlorine. Add a magnet into the bottle and adjust conductivity by the addition of 10gr of artificial sea salts into each sample. AFTER DISSOLVING adjust the pH at 3.5 (± 0.1) by the addition of HCl 1N. Mix the water thoroughly by vigorous stirring while adding the HCl. Add 100 mL of PSM 1% to each water sample.

Stir the samples for 8 hours to allow the viruses to adsorb to the flocks. Use a timer for automatically switch off the stirring and let the flock's sediment by gravity for 8 hours.

Internal control process: (suggested)

Add 1ml of the MS2 phage suspension as a process control to each water sample.

Collecting the flocks, centrifugation and resuspension in phosphate buffer:

Remove the supernatant using a peristaltic pump and a plastic pipette connected to a plastic tube. The supernatant can be removed also by gravity. For spiked samples the supernatant should be collected into a recipient and disinfected according with the internal procedures. In all cases, TAKE CARE not to disturb the pellet. Collect the remaining volume (approximately 500 mL) into a centrifuge bottle (e.g. Scharlab Ref. 195753501). BALANCE THE BOTTLES WITH PSM AT pH 3.5 and centrifuge at 8,000 *xg* 30 min at 4° C. As soon as the centrifuge stops, remove the centrifuge bottles. Very gently pour off and discard the supernatant. Follow appropriate measures for infectious material.

Add 7 mL of phosphate buffer to each centrifuge bottle. Once the flocks have been dissolved, measure and add phosphate buffer to reach a total volume of 10mL. Homogenize the viral concentrate by vortexing and transfer the 10 mL or 5 mL to several Eppendorf which should be frozen until needed in further analysis.

Appendix

Hydrochloric Acid 1 N

- 34.4 mL concentrated hydrochloric acid
- 400 mL deionised water

Measure 400 mL of deionised water in a measuring cylinder and then pour into a clean 500 mL glass bottle. Using a 10 mL disposable pipette add 34.4 mL of concentrated hydrochloric acid. Label with the batch number and the expiration date.

Sodium Hydroxide (1 M/4%)

- 4 g sodium hydroxide
- 100 mL deionised water

Dissolve the sodium hydroxide in the deionised water in a sterile glass beaker. Once dissolved, dispense into 100 mL clean glass bottles. Label with the batch number and expiration date.

Artificial seawater

- 33.33 g Sea Salts (Sigma Ref. S9883)
- 1 L dechlorinated tap water

Add the Sea Salts to water and leave at room temperature overnight to dissolve if is necessary. Shake or swirl container to aid mixing.

Preflocculated skimmed milk (PSM) 1 % (w/v)

- 100 mL of artificial seawater.
- 1 g of skimmed milk (Difco Ref. 232100).

Dissolve using a magnetic stirrer and adjust the pH to 3.5. The flocs should be formed and are clearly visible.

Phosphate buffer

- 1:2 v/v of sterile Na2HPO4 0,2M and NaH2PO4 0,2M at pH 7.5. Label with the batch number and expiry date.

Sodium thiosulfate (10%):

- Dissolve 10 g of sodium thiosulfate into 100 mL of deionised water.