Simultaneous measurement of grazing and viral lysis of phytoplankton

Corina Brussaard, Anne-Claire Baudoux, Anna A. M. Noordeloos, Marcel J. W. Veldhuis

Abstract

An adapted dilution method was used to estimate viral lysis and microzooplankton grazing simultaneously. Parallel dilution experiments were performed with 30 kDa ultrafiltrate (virus and grazer-free diluent) and 0.2 µm filtered seawater (grazer-free, but virus-containing diluent). Specific viral lysis rates were calculated from the difference in P. globosa growth rates between the 2 dilution series after 24 h incubation under natural conditions. The validity of this method was tested using a culture of P. globosa infected with a known P. globosa virus (PgV).

Protocol derived from:

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Guidelines

Study site and sampling. Sampling of the coastal southern North Sea was performed twice a week between March (Day 60) and June (Day 180) in 2003 and 2004 from the jetty of the Royal Netherlands Institute for Sea Research (NIOZ). Because the jetty is located at the outer border of a major tidal inlet, samples were collected on the incoming high tide. Samples containing freshwater run-off (salinity <27‰) were not taken into account (1 out of 46 samples).

Figures:

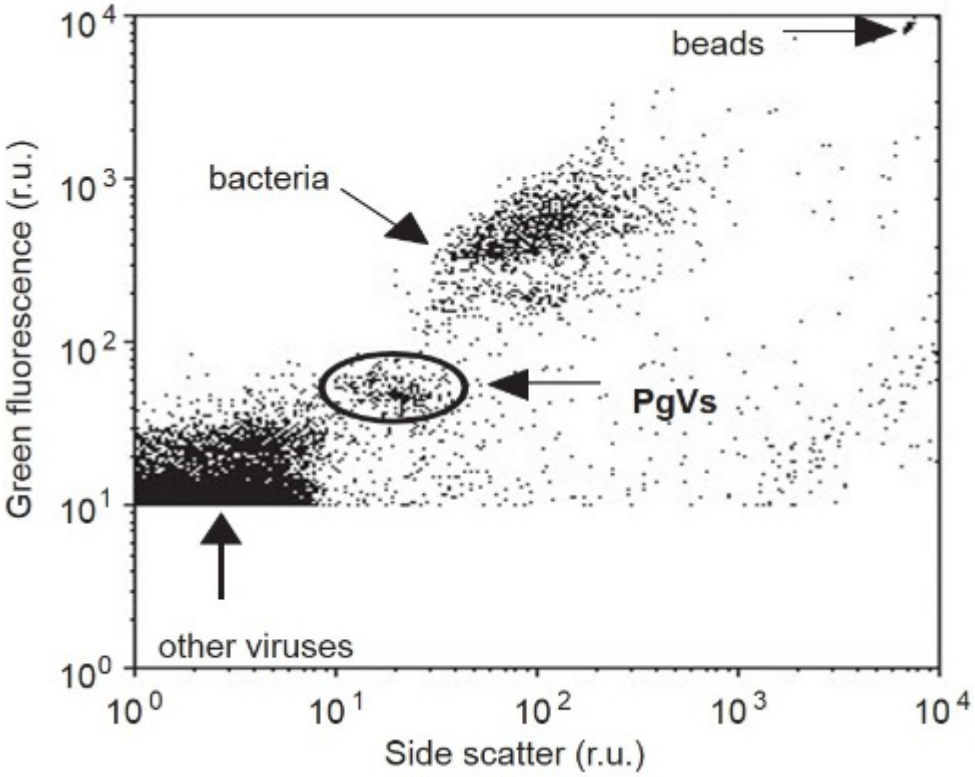

Fig. 1. Flow cytometry signature of viruses infecting *Phaeocystis globosa* (PgVs) from natural seawater. PgVs were detected by their green fluorescence and side scatter upon staining with SYBR Green I. r.u.: relative units

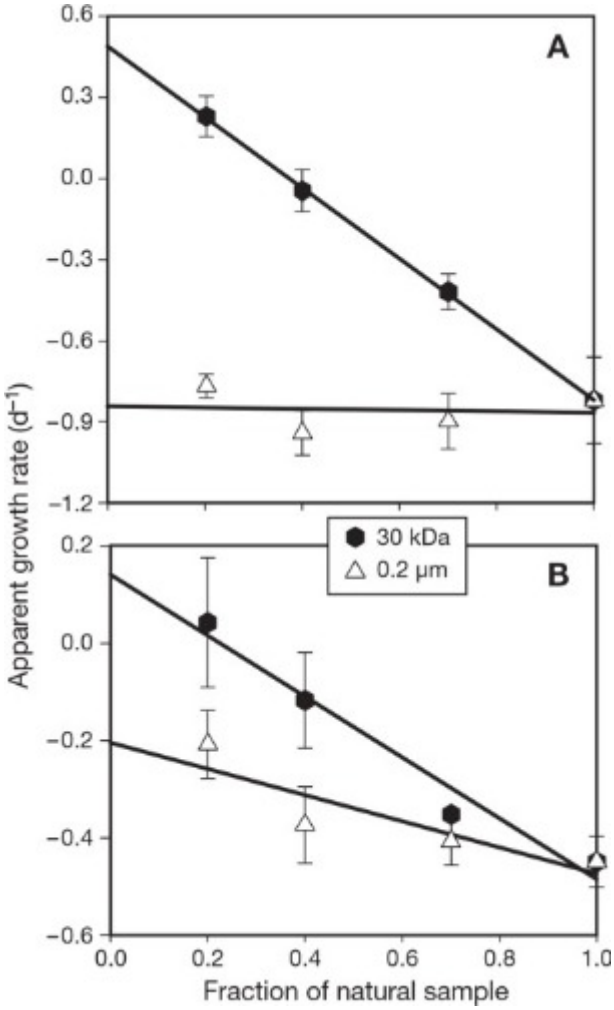

Fig. 2. *Phaeocystis globosa* (Pg-G). Apparent growth rate vs. fraction of natural sample for (A) test experiment using host–virus model system Pg-G–PgV-07T (no grazers added) and (B) typical field sample (Day 132, 2003). Parallel dilution experiments were performed in 0.2 µm filtered seawater (grazer-free, but virus-containing diluent) and 30 kDa ultrafiltrate (virus and grazer-free diluent). Regression coefficient of apparent growth rate vs. dilution factors resulting from 0.2 µm dilution series represents microzooplankton grazing rate, and from 30 kDa series represents microzooplankton grazing plus viral lysis. Viral lysis rates (d^{-1}) were estimated from difference in regression coefficient of the 2 sets of dilutions. For legibility we have averaged the triplicate apparent growth for each dilution level; this does not affect estimated mortality rates

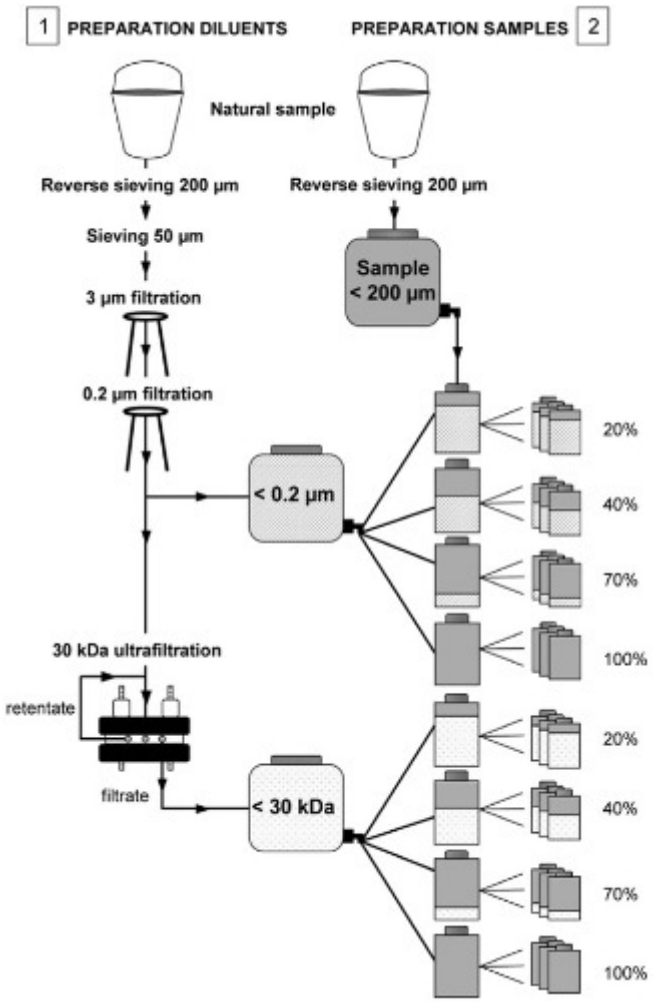

Fig. 3. Experimental design of viral lysis dilution assay (field assay). In Step 1, diluents were prepared ca. 2 h prior to Step 2, which entailed dilution with natural sample collected de novo at high tide. Samples were processed under dim light and at in situ temperature. Sample transfers were performed by siphoning or gentle pumping, avoiding damage to the organisms

Notes:

The viral lysis dilution assay has only been applied to *Micromonas pusilla* (Evans et al. 2003); therefore we checked the validity of this method for *Phaeocystis globosa*. The test experiment was conducted using an exponentially growing *P. globosa* Pg-G (1×10^5 cells ml^{-1}) in combination with the lytic virus PgV-07T (Brussaard et al. 2004) at a multiplicity of infection (MOI) of 10, as determined by the MPN assay (see preceding subsection). In order to simplify the interpretation of the test results no grazers were added; therefore, identical net growth rates were recorded for all dilutions with the 0.2 µm pore-size water, and thus the estimated grazing rate was not significantly different from zero (0.02 ± 0.07 d^{-1}, Fig. 2A). The dilution series with the <30 kDa diluent yielded a regression slope of 1.3 ± 0.07 d^{-1}, which corresponds to the viral lysis rate in this test since there was no grazing. Knowing that *P. globosa* undergoes lysis 14 to 16 h after infection (Baudoux & Brussaard 2005), we conclude that the lysis rate obtained during the 24 h incubation originated from 1 lytic cycle. An independent 1-step lytic growth cycle experiment using the same strain of *P. globosa* and PgV validated the results of the dilution assay. The viral lysis rate (1.4 d^{-1}) calculated from this growth

experiment was comparable to that obtained with the laboratory viral lysis dilution assay (1.3 d^{-1}). These tests demonstrated the utility and validity of the viral lysis dilution assay, allowing this method to be applied in the field to *P. globosa* (Fig. 2B).

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Protocol

Chemical Parameters

Step 1.

Nutrient samples (ca. 5 ml) were gently filtered through 0.2 µm pore-size polysulfone filters (Acrodisc, Gelman Sciences) and stored at –80°C (or 4°C for the reactive silicate) until analysis.

Chemical Parameters

Step 2.

Analyses were performed using a TrAAcs 800 autoanalyzer for dissolved orthophosphate (Murphy & Riley 1962), nitrogen (nitrate, nitrite and ammonium; Helder & De Vries 1979, Grasshoff 1983), and reactive silicate (Strickland & Parsons 1968).

NOTES

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The limit of detection was 0.03 µM for phosphate, 0.1 µM for ammonium, 0.01 µM for nitrite, 0.15 µM for nitrate, and 0.05 µM for silicate.

Chemical Parameters

Step 3.

The concentration of transparent exopolymeric particles (TEP) in µg equiv. gum xanthan (equiv. GX) l^{-1} was measured according to Passow & Alldredge (1995).

Chemical Parameters

Step 4.

Replicate samples (30 to 75 ml) were filtered through 0.4 µm pore-size polycarbonate filters (Poretics).

Chemical Parameters

Step 5.

The particles retained on the filter were stained with 500 µl of a 0.02% solution of Alcian blue prepared in 0.06% acetic acid (pH 2.5).

Chemical Parameters

Step 6.

After staining (<2 s), the filters were rinsed 3 times with Milli-Q (Millipore) to remove excess dye.

Chemical Parameters

Step 7.
The filters were immediately transferred into 20 ml glass tubes and soaked for 3 h in a solution of 80% H_2SO_4 with gentle agitation every 30 min.

DURATION
03:00:00

Chemical Parameters

Step 8.
The samples were analyzed spectrophotometrically at 727 nm (U-3010 Hitachi).

Microbial Abundances

Step 9.
Samples collected for phytoplankton pigments (100 to 1000 ml) were filtered through GF/F glass fiber filters (Whatman) and stored at –80°C.

Microbial Abundances

Step 10.
The extract from the filters was analyzed by high pressure liquid chromatography (HPLC) after extraction in 4 ml of 100% methanol buffered with 0.5 mol l^{-1} ammonium acetate and homogenized for 15 s.

DURATION
00:00:15

Microbial Abundances

Step 11.
The relative abundance of the taxonomic group Prymnesiophyceae (specifically *Phaeocystis globosa* during our study) was determined using CHEMTAX (Mackey et al. 1996, Riegman & Kraay 2001).

Microbial Abundances

Step 12.
Phaeocystis globosa single cells were enumerated in 50 µm-sieved and unfixed samples using a Beckman Coulter XL-MCL flow cytometer equipped with a 488 nm air-cooled laser.

NOTES
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Special care was taken to avoid rupture of *P. globosa* colonies during sieving using a small volume of sample.

Microbial Abundances

Step 13.
The total abundance of *P. globosa* cells (including both single and colonial cells) could be obtained from unfiltered samples that were fixed to a 1% final concentration with formaldehyde:hexamine solution (18% v/v:10% w/v).

NOTES
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Fixation of the sample resulted in the disintegration of the colonial matrix.

Microbial Abundances

Step 14.
These fixed samples were frozen in liquid nitrogen and stored at –80°C until flow cytometry analysis.

NOTES
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Fixation and freezing did not affect the *P. globosa* cell counts.

Microbial Abundances

Step 15.

P. globosa cells were discriminated on the basis of their natural red chlorophyll autofluorescence and forward scatter signal.

Microbial Abundances

Step 16.

The abundance of virus-like particles resembling PgV was determined on glutaraldehyde fixed samples (final concentration 0.5% glutaraldehyde, frozen in liquid nitrogen and stored at –80°C prior analysis) using a Beckton-Dickinson FACSCalibur flow cytometer, with a 15 mW 488 nm air-cooled argon-ion laser according to Brussaard (2004a).

Microbial Abundances

Step 17.

Thawed samples were diluted (dilution factor >10) in 0.2 µm filtered sterile TE-buffer (pH 8) and stained with the nucleic acid-specific dye SYBR Green I at a final concentration of 0.5×10^{-4} of the commercial stock (Molecular Probes).

Microbial Abundances

Step 18.

Putative PgV could be discriminated on the basis of the green fluorescence and side scatter signature (Fig. 1 in guidelines), which was identical to that of PgV isolates from the same geographical location, and kept in culture at the Royal NIOZ (Brussaard et al. 2004).

Microbial Abundances

Step 19.

The abundance of infectious PgV was estimated using the end-point dilution approach (most probable number, MPN; Suttle 1993).

Microbial Abundances

Step 20.

Natural seawater was filtered through a 1 µm polycarbonate filter (Poretics) and serial diluted (8 titers, 5 replicates) with exponentially growing *Phaeocystis globosa* Pg-G (RUG culture collection) and Pg-01MD06 (NIOZ culture collection).

Microbial Abundances

Step 21.

To screen for rare PgV in 2004, an additional natural sample was filtered through a 1 µm polycarbonate filter (Poretics), concentrated ca. 40 times using a VivaFlow 200 ultrafiltration system (Vivascience), and added to a *P. globosa* host culture (20% v/v).

NOTES

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Both *P. globosa* strains originated from the North Sea and were chosen for their different sensitivity to PgV, which was relatively broad for Pg-G and specific for Pg-01MD06 (Baudoux & Brussaard 2005).

Microbial Abundances

Step 22.

The algae were grown in a 1:1 mixture of f/2 medium (Guillard 1975) and enriched artificial seawater (ESAW) (Harrison et al. 1980, Cottrell & Suttle 1991), completed with Tris-HCl and Na_2SeO_3 (Cottrell & Suttle 1991).

Microbial Abundances

Step 23.

The dilution series were incubated for 10 d at 15°C under a light:dark cycle of 16:8 h at 100 µmol quanta m^{-2} s^{-1}.

Microbial Abundances

Step 24.

Algal growth was monitored via in vivo chlorophyll fluorescence using a Turner Designs fluorometer and compared to noninfected controls.

Microbial Abundances

Step 25.

Those dilutions that showed signs of cell lysis were scored positive when PgV proliferation could be confirmed (using flow cytometry as described above).

Microbial Abundances

Step 26.

The positive scores were converted to abundance of infective PgV using an MPN assay computer program (Hurley & Roscoe 1983).

Loss parameters of Phaeocystis globosa

Step 27.

Total cell lysis rates of *P. globosa* (d^{-1}) were estimated using the dissolved esterase activity (DEA) assay described in Brussaard et al. (1996a) and adapted by Riegman et al. (2002).

Loss parameters of Phaeocystis globosa

Step 28.

Particulate esterase activity (PEA) was obtained by subtracting the DEA (0.2 µm pore-size filter) from the total esterase activity (unfiltered natural sample).

Loss parameters of Phaeocystis globosa

Step 29.

The DEA was corrected for non-enzymatic hydrolysis of the substrate, as measured in natural samples filtered through 10 kDa (PES Vivaspin, Vivasciences), and for a decay of esterase activity in seawater using a half-life time of 48 h (Riegman et al. 2002).

Loss parameters of Phaeocystis globosa

Step 30.

The *P. globosa*-specific PEA was calculated by multiplying the total PEA by the contribution of *P. globosa* to total chlorophyll-based CHEMTAX pigment analysis described in an earlier subsection (Brussaard et al. 2004).

NOTES

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Data points were occasionally omitted when an unrealistically high lysis rate was obtained from the ratio of low produced DEA to low *P. globosa*-specific PEA. This may occur at the onset of the bloom when *P. globosa* biomass is still low.

Loss parameters of Phaeocystis globosa

Step 31.

Virally induced mortality of *Phaeocystis globosa* single cells was estimated using the viral lysis dilution assay according to Evans et al. (2003).

Loss parameters of Phaeocystis globosa

Step 32.

Parallel dilution series of natural seawater was performed with 0.2 µm filtered natural sample (Poretics, Millipore) to obtain microzooplankton grazing rate (Landry & Hassett 1982), and with a 30 kDa filtered natural sample (polyether sulfone membrane, Pellicon filtration system, Millipore) to

obtain grazing and viral lysis rates.

Loss parameters of Phaeocystis globosa

Step 33.

Viral lysis rates were determined from the difference between the 2 dilution series.

Loss parameters of Phaeocystis globosa

Step 34.

The experimental design of the dilution assay for field samples is shown in Fig. 3 (guidelines).

Loss parameters of Phaeocystis globosa

Step 35.

All materials (carboys, tubing, bottles) used for this assay were cleaned for 24 h with 0.1 N HCl, after which they were rinsed 3 times with Milli-Q and once with the sample.

DURATION

24:00:00

Loss parameters of Phaeocystis globosa

Step 36.

To prevent losses of virus, grazers or disruption of *Phaeocystis globosa* colonies, sieving and filtration were performed with special care by siphoning and avoiding air bubbling.

Loss parameters of Phaeocystis globosa

Step 37.

Polycarbonate Poretics filters (47 mm, Millipore) were exclusively used and replaced frequently during the filtration to avoid loss of viral infectivity and abundance (Suttle et al. 1991).

Loss parameters of Phaeocystis globosa

Step 38.

Samples were processed under dimmed light (to prevent light stress) and in situ temperature (4 to 18°C).

Loss parameters of Phaeocystis globosa

Step 39.

The seawater used for the dilution (10 l) was collected and processed ca. 2 h before high tide in order to minimize the handling time of the natural water to be diluted.

Loss parameters of Phaeocystis globosa

Step 40.

The 10 l sample was pretreated by reverse sieving through 200 and 50 µm mesh (20 cm diameter) to remove larger grazers and *P. globosa* colonies.

Loss parameters of Phaeocystis globosa

Step 41.

Subsequently, the sample was filtered through 3 and 0.2 µm pore-size filters.

Loss parameters of Phaeocystis globosa

Step 42.

A 5 l aliquot of the 0.2 µm filtrate was used for generating the 0.2 µm dilution series.

Loss parameters of Phaeocystis globosa

Step 43.

The remaining 5 l were ultrafiltrated through 30 kDa and used as diluent for the 30 kDa dilution series.

Loss parameters of Phaeocystis globosa

Step 44.

At high tide, 20 l of natural seawater were collected de novo.

NOTES

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The salinity of the 2 batches of seawater used for the experiment was measured and comparable in all cases (difference <0.5‰).

Loss parameters of Phaeocystis globosa

Step 45.

The sample was reverse sieved through 200 µm mesh to remove mesozooplankton, and immediately used to set up 4 levels of dilution (20, 40, 70 and 100% sample) in 2 l polycarbonate bottles that already contained the 0.2 µm or the 30 kDa diluents.

Loss parameters of Phaeocystis globosa

Step 46.

From the 2 l dilution bottle, 3 incubation bottles (250 ml polycarbonate bottles) were carefully filled by siphoning and 5 ml subsample were taken (time, t = 0 h).

Loss parameters of Phaeocystis globosa

Step 47.

These incubation bottles were refilled to the top with the original dilution waters (remaining from the 2 l bottles) in order to avoid any air bubbles being trapped inside upon closure.

Loss parameters of Phaeocystis globosa

Step 48.

All bottles were incubated at near-surface depth (ca. 1 to 2 m) under natural light and temperature conditions in a basket in the NIOZ harbor (protected from wave-motion).

Loss parameters of Phaeocystis globosa

Step 49.

Another sample of 5 ml was taken after a 24 h incubation period.

DURATION

24:00:00

Loss parameters of Phaeocystis globosa

Step 50.

The set-up of the laboratory assay was similar to the field assay with the exception that a *Phaeocystis globosa* culture free of virus and grazers was used.

Loss parameters of Phaeocystis globosa

Step 51.

Viruses infecting *P. globosa* (0.2 µm filtered PgV-07T lysate, Poretics filters, Millipore) were added (MOI = 10) to the diluent directly after the 0.2 µm filtration step and to the *P. globosa* culture just prior to dilution.

Loss parameters of Phaeocystis globosa

Step 52.

The P. globosa culture that had to be diluted was infected just before setting up the dilutions in the 2 l bottles.

Loss parameters of Phaeocystis globosa

Step 53.

All bottles were incubated under the same growth conditions as the host culture (15°C, light:dark cycle of 16:8 h, 100 µmol quanta m^{-2} s^{-1}).

Loss parameters of Phaeocystis globosa

Step 54.

For both field and laboratory assays, *Phaeocystis globosa* single cells were enumerated directly upon

sampling after gentle sieving through 50 µm mesh-size using a Beckman Coulter XL-MCL flow cytometer (3 replicates of each sample).

Loss parameters of Phaeocystis globosa

Step 55.

The apparent growth rate (µ, d^{-1}) of *P. globosa* single cells was calculated for each sample from the changes in abundance during the incubation according to the equation:

$$\mu = \ln N_{t24} - \ln N_{t0}$$

where N_{t0} and N_{t24} are the abundance of *P. globosa* single cells at t = 0 and 24 h, respectively.

Loss parameters of Phaeocystis globosa

Step 56.

A typical field example is presented in Fig. 2B (guidelines). The regression coefficient of apparent growth rate vs. dilution factors for the 0.2 µm dilution series represents the microzooplankton grazing rate (M_g), whereas the regression coefficient resulting from the 30 kDa series represents both microzooplankton grazing and viral lysis ($M_{(g+v)}$).

Loss parameters of Phaeocystis globosa

Step 57.

Subsequently, mortality rate due to viral lysis (M_v) was calculated as:

$M_v = M_{(g+v)} - M_g$.

NOTES

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M_g, $M_{(g+v)}$ and their respective standard errors (SE_g and $SE_{(g+v)}$) were calculated using Sigma plot software. The standard error of M_v was calculated as the squared root of the sum of squared SE_g and $SE_{(g+v)}$