MEASURING AND MODELLING THE DYNAMIC RESPONSE
OF REMOTE MOUNTAIN LAKE ECOSYSTEMS TO
ENVIRONMENTAL CHANGE

A programme of **MO**untain **LA**ke **R**esearch

MOLAR

MICROBIAL (PELAGIC) FOOD WEBS - 2nd LEVEL

FIELD AND LABORATORY PROTOCOL FOR EXPERIENCED LABS
Work Package 1.

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Microbial (Pelagic) Food Webs - 2nd Level

Field and laboratory protocol for experienced labs, WP1.

1. Objective

Assessment of fluxes among the pelagic components. The protocol is not strict and will be adapted according to specific conditions of particular lakes. Alternative methods are recommended. The measurement of fluxes will be performed only in several lakes of WP 1: Ovre Neadalsvatn, Lochnagar, Redo, La Caldera, Gossenkoellesee, Jorisee, Dlugi staw, Starolesnianske.

For constructing a model (carbon flow chart) of pelagic food webs, which should characterize "an average situation in the lake during the second half of ice-free period", the data of biomasses (1st level) and fluxes must be generated *from particular depths and days simultaneously.* The measurements should be carried on during several short periods of intense investigations - e.g. three days in one week, and during 2 - 3 weeks per two ice-free seasons, if possible.. It is not planned to construct a model of seasonal dynamics. This could not be achieved in the whole set of remote lakes included in WP1. Just two lakes of the set have a well equiped laboratory (with electricity) located close to the lake, where such measurements could be carried on regularly during the whole season.

2. Phytoplankton primary production

2.1 Principle

^{14}C-method is used. Incorporation of ^{14}C by phytoplankton cells as well as bacterial incorporation of photosynthetically produced extracellular organic C, plus concentration of photosynthetically produced and released organic C not utilized by bacteria, is measured. Only the photosynthetically produced carbon, which was released by respiration during the incubation could not be assessed. If possible, the fractions of primary production are separated by differential filtration:

a. Incorporation by phytoplankton on 0.8 - 2 μm filter (polycarbonate filters: Nuclepore, Poretics, are necessary, which retain phytoplankton but not bacteria)

b. incorporation by bacteria (from the filtrate of A) on 0.2 μm filter

c. labelled organic carbon in the filtrate of B.

Depending on the structure of phytoplankton at particular lake and date, the appropriate porosity of algal filter (A) should be chosen. In some cases, separation of phytoplankton and bacteria may be impossible due to overlap in the sizes of phytoplankton and bacteria or due to very low primary production. Then the sum of "particulate" production should be measured (A+B) on 0.2 μm filters of any material (polycarbonate, glass-fiber, cellulose-acetate etc.).

In field conditions when differential filtration would be difficult, total labelled organic carbon might be measured (A+B+C) in a subsample before filtration, instead of using filtrate (C).

2.2 Sampling and incubation strategy

Thermal stratification of the lake and the depth of euphotic layer (2 x Secchi depth) should be determined. The layers sampled should include 0.5 m plus four other layers within the euphotic layer.

In the lakes with not pronounced stratification or in very shallow lakes, the number of layers sampled may be reduced.

Note that primary production, concentration of chlorophyll, bacterial production and elimination are to be measured at identical depths.

All samples should be exposed in the same layers, from which they were sampled, for 2 - 4 h (preferentially 10 a.m. to 2 p.m.). Actual values of primary production, valid for particular light and temperature conditions in each depth during measurement, are thus obtained (similarly as the values of bacterial production and elimination). These data cannot be used for the estimation of primary production in the whole lake and whole season. If an estimate of total production in the lake is desirable, primary production from each sampled depth should be measured at different light intensities. The procedure is described in a separate Protocol for measuring photosynthetic rates by using the ^{14}C method and estimation of primary production (L. Camarero). Using this procedure, it is necessary to have data on primary production extrapolated to the actual light intensity and temperature in the layers where bacterial production and elimination is determined.

2.3 Incubation

- Collect a sample from each depth

- For each depth, fill two dark and two light quartz-glass or PET bottles (100-250 ml) plus one bottle for C_{inorg} determination, plus another bottle for Chl.*a* determination (for the determination of C_{inorg} and Chl.*a* see the last two sections

- Keep the bottles in dark (e.g. in insulated plastic container) until start of incubation Dispense 0.5 Mbq of ^{14}C-bicarbonate in small volume (50-200 ml) per bottle. Amount of radioactivity can be adjusted (depending on expected rate of photosynthesis and C_{inorg} concentration) to get appropriate values of d.p.m./filter

- Expose bottles in the depths of sampling, after exposition stop incubation by addition of formalin (1 % final concn). Formaldehyde can be avoided if the time period between incubation and filtration is very short

- Store bottles in dark and cold until filtration

2.4 Filtration and C_{org} separation

- From each bottle remove a 1-ml subsample for determination of total activity and transfer it into scintillation vial containing 1 ml of β-phenetylamine (or Carbosorbe) - (T)

- Filter a defined volume (50-100 ml) of incubated subsamples through algal filter, collect the filtrate. The porosity of filter must be chosen according the locality sampled, the filter should retain phytoplankton but not bacteria - see above:Principle - (A).

- Filter the collected filtrate through 0.2-mm (bacterial - B) filter, collect the filtrate. (alternatively: only one filter A+B from a non-filtered sample will be filtered - see above: Principle)

- Vacuum must not exceed 20 kPa (0.2 atm) during the filtrations

- To the 10 - 20 ml aliquote of the 0.2-mm filtrate add in a small volume (10-100 ml) HCl to the final concentration of 0.01 N (C)
  (alternatively: use an aliquote from non-filtered sample - see above: Principle - A+B+C)

- Bubble the acidified filtrate with air for 60 min to remove inorganic ^{14}C. Alternatively, let aliquotes stand in open vials overnight.

- Neutralize the aliquote with NaOH added in a small volume

- Transfer 5-10 ml of the neutralized filtrate into scintillation vial, add appropriate volume of the liquide scintillator capable of mixing with water

- Determine ^{14}C radioactivity (d.p.m.) of all filters, and of neutralized aliquotes.

2.5 Calculations

- Depending on the volumes incubated, volumes filtered, and volumes used for aliquotes calculate for each bottle:

  A = radioactivity retained on the algal filter per liter of original sample
  B = radioactivity retained on the bacterial filter per liter of original sample
  C = radioactivity of acidified filtrate (dissolved C_{org}) per liter of original sample
  T = total added radioactivity per liter of original sample

  (alternatively, you may have either:
  A, B, (A+B+C), T .....then C can be calculated
  or: (A+B), C, T ..... then B cannot be calculated)

- Calculate relative values of primary production and exudation as $A_{rel} = A/T$ etc.

- Correct values in light bottles for (blank) values in dark bottles $A_{rel} = A_{rel}(light) - A_{rel}(dark)$

- Using values of C_{inorg} calculate absolute values of primary production and of exudation:
  $A_{abs}[\mu gC \cdot l^{-1} \cdot h^{-1}] = (C_{inorg} + {}^{14}C_{inorg}) \prec 1.06 \prec A_{rel}$
  $B_{abs}[\mu gC \cdot l^{-1} \cdot h^{-1}] = (C_{inorg} + {}^{14}C_{inorg}) \prec 1.06 \prec B_{rel}$
  etc.

where C_{inorg} is concentration of dissolved inorganic carbon and $^{14}C_{inorg}$ is the final concentration of ^{14}C
bicarbonate added (it can be neglected if $C_{inorg} >> {}^{14}C_{inorg}$)

A+B+C	=	**gros primary production**
A	=	**net primary production**
B+C	=	**extracellular production (exudation)**
B	=	**part of extracellular production used by bacteria**

2.6 Inorganic carbon determination

Inorganic carbon (DIC) can be determined directly by using an inorganic carbon analyzer or estimated from pH and alkalinity (using Gran titration). It is important to prevent samples

from any contact with air during storage before DIC analysis or pH determination (to avoid any CO_2 exchange).

See Protocol for measuring photosynthetic rates by using the ^{14}C method and estimation of primary production (L. Camarero) for detailed description of the procedure for DIC calculation based on pH and akalinity.

2.7 Chlorophyll determination

Spectrophotometric method is used.
Lakewater is filtered through GF/C filter (1 - 3 l according to Chl concn) and analyzed immediately or freezed.

Extraction of pigments is performed with 4 - 5 ml acetone 90% by grinding the filter or using a sonicator. Extraction vial should be cooled and extraction time minimized to avoid evaporation of acetone. After grinding, the remains of the filter are removed by centrifugation or filtration through a fiberglass filter. The supernatant (or the first filtrate) is then filtered through 0.1 μm Anopore filter for a complete clarification. Turbidity is checked by measuring the absorbance at 750 nm (it should not exceed 0.002).

Absorbance is then read at 663, 647 and 630 nm.
Chlorophyll.*a* concentration is computed with the equation (Jeffrey 🕮 Humphrey 1975, *Biochem. Physiol. Pflanzen* **167**: 191-194):

$$\text{Chl.}a\ [\mu g \cdot l^{-1}] = [11.85\ (A663 - A750) - 1.54\ (A647 - A750) - 0.08\ (A630 - A750)] \times \text{VOLacet[ml]} / \text{VOLfilt [l]} \times \text{lc}$$

where:
VOLacet = volume of the acetone extract
VOLfilt = volume of lakewater filtered
lc = length of the spectrophotometric cuvette in cm

3. Bacterial production

3.1 Principle

Incorporation of (i)^{3}H-thymidine or (ii)^{3}H-leucine by bacteria is used for estimating production: (i) external thymidine is supposed to be taken up at a rate proportional to the rate of nucleotides synthesis (de novo synthesis of thymidine by the cells is neglected), (ii) leucine is supposed to be taken up at a rate proportional to the rate of protein synthesis.

3.2 Sampling

- Determine the thermal stratification of the lake under study and the depth of euphotic layer(2 × Secchi depth).

- Make a decision about the depths to be sampled (0.5 m + four further depths within the euphotic layer). Note that bacterial production and primary production are to be measured at identical depths. In the lakes with not pronounced stratification of bacterial parameters, the number of depths sampled may be reduced.

- Collect water samples, avoid change of temperature of samples between sampling and the beginning of incubation (if necessary, keep water samples in thermally insulated boxes, bottles, etc.). Begin the incubation within 60 min after sampling.

3.3 Incubation (the same for ^{3}H-thymidine and ^{3}H-leucine)

- For each sample, prepare 6 incubation vials (e.g. washed plastic scintillation vials), three of them will be blanks (in the case of uniformly low blank values the number of blanks can be reduced).

- To the blank vials, dispense appropriate volume of 40-% formaldehyde (50 ml per 1 ml of the incubated subsample; final concentration 2 %)

- To all vials, add a small volume (10-100 ml) of working solution of ^{3}H-thymidine (^{3}H-TdR) or ^{3}H-leucine (^{3}H-Leu). The same volume add also directly into scintillation vial (in duplicate) for determination of the total activity (and concentration) of ^{3}H-TdR or ^{3}H-Leu present during the incubation. The added volume depends on the concentration of the working solution of ^{3}H-TdR or ^{3}H-Leu, on the incubation volume and on the required final concentration (see the last section).

- **The final concentration**of both ^{3}H-thymidine and ^{3}H-leucine is **25 nmol·l^{-1}.** A lower concentration can be used only if it has been demonstrated (in preliminary saturation experiments) that incorporation is already saturated at the lower concentration (to check saturation, use e.g. concentrations of 2-5-10-15-20-25-50 nmol·l^{-1} of ^{3}H-TdR or ^{3}H-Leu).

- Start the incubation by the addition of a 10-ml subsample of water sample. The volume can be increased to get higher filter-counts.

- Incubate samples **in the dark at *in-situ* temperature**. The samples where temperature differs less than by 2 °C, may be incubated at the same temperature. For each incubation temperature use a separate incubation bath with temperature adjusted to the temperature of the sample (e.g. polystyrene box filled with water taken from the sampling depth can be used). Alternatively, subsamples can be incubated directly *in-situ*, in the sampling depth .

- Incubate for **30-60 minutes.** The incubation period can be extended (up to several hours) only if it has been demonstrated (in preliminary experiments) that the time course of incorporation is linear over the whole period of incubation.

- Stop the incubation by the addition of 40-% neutral formaldehyde (the same volume as it has been used in blanks).

3.4 Extraction

- Between the end of incubation and extraction, keep the subsamples in cold (~ 4 °C). Extraction should be completed as quickly as possible, at latest during the next day.

- There are two extraction procedures:

  a. *Cold TCA extraction* (specific for macromolecules = DNA + RNA + proteins): This procedure can be used for both ^{3}H-TdR and ^{3}H-Leu incorporation, because the incorporation of the radioactivity from ^{3}H-TdR into proteins and from ^{3}H-Leu into nucleotides is low during the short incubation times used (£60 min).

b. *Hot TCA extraction* (specific for proteins) may be alternatively used for ^{3}H-Leu incorporation .

3.4.1 Cold TCA extraction

- Filter subsamples through 0.2-mm polycarbonate filter (Nuclepore, Poretics etc. - using the cellulose-nitrate or cellulose-acetate filters may result in a very high blank values!).
- Rinse filters 10 × with 1 ml of ice-cold 5-% trichloroacetic acid (TCA).
- Vacuum must not exceed 20 kPa (0.2 atm) during the filtration and the TCA extraction.
- Place filters in scintillation vials, add scintillation cocktail suitable for tritium counting.
- Count for ^{3}H.

3.4.2 Hot TCA extraction

- Use 5-% TCA instead of formaline for blank vials and for incubated subsamples.
- Heat the 5-% TCA killed subsamples to 80 °C for 15 min.
- Filter subsamples through 0.2-mm polycarbonate filters
- Vacuum must not exceed 20 kPa (0.2 atm) during the filtration and the TCA extraction.
- Rinse filters 2 × with 3 ml of ice-cold 5-% TCA and then 2 × with 2 ml of ice-cold 80-% ethanol.
- Place filters in scintillation vials, add scintillation cocktail suitable for tritium counting.
- Count for ^{3}H.

3.5 Calculation of the incorporation rate of ^{3}H-TdR or ^{3}H-Leu

- Correct d.p.m. values (d.p.m. per filter) for blanks
- Convert d.p.m. values to pmol·l^{-1}·h^{-1} of ^{3}H-TdR or ^{3}H-Leu

$$Incorporation\,rate\left[pmol\,/\,l.h\right]=\frac{d.p.m./\,filter\,\left(corrected\,for\,blank\,values\right)}{60\times Spec.Act.\left[TBq\,/\,mmol\right]\times Incub.volume\left[ml\right]\times Time\left[h\right]}$$

Example:

d.p.m./filter: 5 000
Specific activity: 1.81 MBq·mmol^{-1}
Incubation volume: 10 ml
Time: 60 min

Incorporation rate = 5 000 / (60 × 1.81 × 10 × 1) = 4.6 pmol·l^{-1}·h^{-1}

- Bacterial production in terms of cells produced per unit of volume and time can be calculated using appropriate conversion factor. The conversion factor should be determined experimentally. Alternatively, for ^{3}H-TdR theoretical conversion factor of 2×10^{18} cells × (mol of incorporated ^{3}H-thymidine)$^{-1}$ can be used.

3.6 Preparation of work solutions of 3H-thymidine and 3H-leucine

- In sterile vessel, dilute original ^{3}H-TdR or ^{3}H-Leu preparation with bidistilled water to achieve appropriate concentration (calculation of the dilution factor see below).

- By means of a syringe assembled with a sterile disposable filter holder with 0.2-mm filter, dispense appropriate portions of the diluted ^{3}H-thymidine into sterile plastic tubes (adjust volume of ^{3}H-thymidine in vials to that which will be used in one experiment).

- Store at -20 °C.

Calculation of the dilution factor:
Dilution factor depends on radioactive concentration and specific activity of the original ^{3}H-TdR or ^{3}H-Leu preparation, on the volume in which you want to add work solution into incubation vials, on incubation volume and on the desired final concentration of ^{3}H-thymidine during incubation:

$$Dilution\ factor = \frac{Rad.concn.\left[MBq/ml\right] \times Added\ volume\left[\mu\ell\right]}{Spec.act.\left[TBq/mmol\right] \times Incubation\ volume\left[ml\right] \times Final\ concn.\left[nmol/l\right]}$$

Example (^{3}H-TdR):

Added volume:	50 ml
Specific activity:	1.81 MBq·mmol^{-1}
Radioactive concentration of original preparation:	37 MBq·ml^{-1}
Incubation volume:	10 ml
Final concentration:	25 nmol·l^{-1}

Dilution factor = 37◄50/(1.81◄10◄25) @ 4.1

Preparation of the work solution:
1 : 4.1 ═ e.g. 1 ml of original preparation of ^{3}H-TdR + 3.1 ml of bidistilled H_2O

4. Estimation of bacterivory

Fluorescently labelled bacteria (FLB) stained by DTAF will be used (Sherr 🕮 Sherr 1993). Protozoa, especially HNF and ciliates, are recognized as major consumers of bacteria in most of freshwater pelagic ecosystems. Since both these protozoan groups are strongly size-selective, size class distribution of FLB offered should mimic very tightly the size class distribution of bacteria in a studied lake ecosystem. There are basically two approaches available:

4.1 Time-integrated grazing impact of all (nano- and micro-) bacterivores

This approach is based on disappearance rate of FLB (constituting cca 1 - 5 % of natural bacterial concentration) with time. This method will be more convenient for those who do not have enough experience with measurements of direct uptake of FLB by protozoans (2nd approach). Water samples (at least 250 ml) with an addition of FLB will be exposed in duplicates *in situ* or at least at *in situ* temperature for 1-2 days. Subsamples (20-30 ml) will be taken at the start and end of the exposure and preserved by formaldehyde (2% final concn). In 5-10 ml subsamples filtered through 0.2 ✪m pore-size black filters, FLB will be enumerated using the same filter set as for acridine orange. Along with the FLB counting, natural bacterial concentration in the same subsamples has to be quantified (DAPI staining, see procedure for bacterial counting). To calculate the rate of cell disappearance, a linear model which takes into account also changes in natural bacterial abundance will be used.

$$G = (F_0 - F_T) \wp \frac{N}{F} \quad \text{where} \quad N = \frac{N_0 + N_T}{2} \quad \text{and} \quad F = \frac{F_0 + F_T}{2}$$

G ..... bacteria grazed during a period T
N_0 and N_T ..... natural bacterial abundance at time 0 and time T, respectively
F_0 and F_T ..... FLB abundance at time 0 and time T, respectively.

For details see Salat and Marasse (1994).

Elimination rate (e) can be estimated as G/N_0 per time.
An estimate of bacterial production rate (p) could be calculated as follows:

$$p_{ap} = \frac{\ln N_T - \ln N_0}{T} \qquad p = p_{ap} + e$$

where:

p_{ap} = apparent production rate ("net" rate, if elimination is not considered)

4.2 Direct uptake of FLB

Experiments are conducted in at least 250 ml samples using 30 min. incubation. Tracer amount of FLB added should account for cca 10-20% of natural bacterial abundances. Subsamples (30-50 ml) from time zero and 30 min. after tracer addition are fixed by adding 0.5 % of alcaline Lugol solution immediately followed by 2% formaldehyde (final concn) and several drops of 3% sodium thiosulphate to clear the color of Lugol (Sherr & Sherr 1993). To determine flagellate and ciliate grazing rates, at least 50 HNF and 30 ciliates should be inspected per sample fixed after 30 min (the same procedure as the determination of HNF numbers, using DAPI stain and switching between filter sets for DAPI and for acridine orange-DTAF). Samples from zero time are also inspected to avoid a potential bias due to attachment of non-ingested tracers on protozoan surfaces. To estimate total protozoan grazing rate, average uptake rates of HNF and ciliates are multiplied by their *in situ* abundances.

* Alkaline Lugol: dissolve 10 g of KI in 20 ml dist. water, then add 5 g of cryst. iodine (sol. A), dissolve 5 g of sodium acetate in 50 ml dist. water (sol. B), then mix A + B

These two methods do not provide same results. During frequent measurements throughout the season, at average, the estimates of bacterial elimination by grazing based on both methods are comparable, though they differed (to both sides) in particular samples. The same is valid for a comparison of bacterial production based on thymidine uptake and with the estimate based on changing bacterial numbers (see approach 1).

References

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