Experimental Protocol for monitoring the impact of tropospheric ozone on white clover

Malé Declaration Network
http://www.rrcap.unep.org/issues/air/maledec/
http://www.york.ac.uk/inst/sei/rapidc2/male.html
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Attention: Mr. Mylvakanam Iyngararasan

Please contact a member of RAPIDC (Regional Air Pollution in Developing Countries) if you require further information or have any difficulties with any aspects of this protocol:

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Acknowledgement: This protocol is partly based on the experimental protocol used for several years within the ICP Vegetation network (International Cooperative Programme on Effects of Air Pollution on Natural Vegetation and Crops) (cf. Mills et al., 1997) and has been amended for application within Malé countries.

CONTENTS PAGE

1. Introduction

In accordance with the overall aim of RAPIDC (Regional Air Pollution in Developing Countries), i.e. "to facilitate the development of agreements and protocols and methods to implement measures which prevent and control air pollution in developing countries", the Swedish International Development Co-operation Agency (Sida) agreed to fund a third RAPIDC project phase with a special focus on assessing the impacts of tropospheric ozone on crops in South Asia and southern Africa.
It has been widely acknowledged that large parts of Asia and Africa experience a distinct increase in air pollution emissions both on a local and regional level due to enhanced industrial growth and population density, which leads to increased emissions from household (burning of fossil fuels or wood), transport and industrial activities. Due to its high phytotoxicity and occurrence not only in urban but in particular periurban and rural, agricultural areas, the secondary air pollutant ozone has been identified as a main threat for crop production. In fact, various surveys from different parts of the world have shown that increased ozone concentrations result in foliar injury and biomass reduction of sensitive crop species, such as wheat, rice, beans and potatoes (e.g. Tingey et al., 1993; Fuhrer et al., 1997; Agrawal et al., 2003, 2006).

In order to assess the biological impacts of increased ozone concentrations on plants and to identify areas at risk in South Asia and southern Africa, a biomonitoring campaign using ozone-sensitive and ozone-resistant white clover genotypes (*Trifolium repens* cv. Regal) was suggested for the third RAPIDC funding phase (2005-2008). This campaign is believed to have the potential to verify that impacts of tropospheric ozone on crops are actually occurring in the field, as predicted by modelling-based risk assessments (e.g. van Tienhoven, 2006).

The reason for using two genotypes of white clover, a plant extensively used in meadows in temperate climates but not native Malé countries, is the high degree of standardisation, reliability based on extensive experience and good reputation of this bio-monitoring system. It was initially developed in the humid subtropical climate of North Carolina, U.S.A. (cf. Heagle et al., 1994) and has been used extensively and successfully within the ICP Vegetation (International Cooperative Programme on Effects of Air Pollution on Natural Vegetation and Crops) in Europe. The principle idea behind this bio-monitoring concept is that the difference in plant foliar injury as well as the biomass ratio between the ozone-sensitive and ozone-resistant clover genotypes can directly be related to the prevalent ozone concentrations during the exposure time.

These exposure-response relationships helped in the past to define critical levels to protect sensitive vegetation against ozone injury. More precisely, a short-term critical level of a VPD-modified AOT30 (Concentration accumulated over a threshold ozone concentration of 30 ppb under consideration of the modifying influence of the vapour pressure deficit (VPD); cf. Mills, 2004) of 0.16 ppm.h accumulated over 8 days preceding the occurrence of visible foliar injury is applied to protect vegetation against this ozone-induced injury, whereas an AOT40 of 3 ppm.h accumulated over a three-month growing season is believed to protect semi-natural vegetation, such as white

clover, against growth reduction in perennial and annual species. Details of the derivation and application of dose-response relationships can be found in Mills, 2004.

However, the calculation of AOTX values requires half-hourly or hourly ozone concentration records, which will not be available for the RAPIDC biomonitoring campaign. Instead, passive samplers providing four-weekly integrated ozone mean concentrations will be used, because they are cheap, easy to deploy and don't require a power supply. Eventually, the extent of foliar injury and biomass reduction will be related to the four-weekly mean ozone concentrations derived from the use of these passive samplers.
Furthermore, the micro-meteorological parameters air temperature and relative humidity will be recorded simultaneously using micro-dataloggers, which will permit the consideration of VPD (the vapour pressure deficit of the air; an important modifier of stomatal conductance and thus of gas exchange in plants) in the analysis of the biological results obtained.

Finally, it has to be pointed out that the use of white clover genotypes should be considered as a first step to assess whether or not any adverse effects of ozone on plant health are detectable. This experiment benefits from its high standardisation (genetically uniform plants, automated water supply, standardised soil and fertilizer), which enables a direct comparison of the impact of ozone on plants in different parts of Asia and Africa. In addition, this experiment will function as a cohesive experimental initiative the participants of the Malé network can identify with. However, in the long run participants will be encouraged to add species and cultivars of high local interest, i.e. representing the respective staple crop, to this biomonitoring initiative. Potential candidates know to be ozone-sensitive are for example beans, millet, rice, sorghum, maize etc.

In conclusion, the aims of the Malé/RAPIDC biomonitoring campaign are:

- to conduct coordinated, standardised experiments to determine the effects of ozone pollution on crops (and (semi-)natural vegetation) in South Asia
- to evaluate the results of a provisional risk assessment
- to establish a scientific crop-effect community within the Malé network with a special focus on experimental initiatives, such as biomonitoring, chemical protectant and gradient studies
- to give experimental evidence as a basis for a survey on socio-economic implications of crop yield losses related to air pollution

This Experimental Protocol provides the standardised methodology to be adopted by participants in the Malé network for monitoring the impact of ozone, expressed as foliar injury and biomass reduction, on white clover (*Trifolium repens* L. cv. Regal).

2. OZONE CONCENTRATION MEASUREMENTS (PASSIVE SAMPLERS)

To aid the interpretation of the biomonitoring study results, i.e. to be able to relate the apparent foliar injury and biomass reduction to the pollution climate, it is important to record the ozone concentration at or next to the experimental sites.
Ideally, continuous measurements of ozone should be made at the experimental sites using analyzing methods such as gas-phase chemiluminescent, gas-solid chemiluminescent or UV photometric. However, if continuous analyzers are not available (which will be the case at most sites), the use of passive samplers is recommended. Passive sampling is an inexpensive, reliable and simple ozone measurement technique that doesn't require electricity and is therefore thought to be ideal for use within the RAPIDC biomonitoring activities. The sampling technique is based on molecular diffusion of gases. The gas molecules diffuse into the sampler where they are quantitatively collected on an impregnated filter or an adsorbent material. Hence, this method gives concentration values integrated over time, in this case the mean value of **four-weekly** ozone concentrations. For details on passive samplers, please check Appendix 1.

Instructions for outdoor sampling with ozone passive samplers (as defined by IVL, Sweden)

Figure 1 Passive sampler as distributed by IVL

The passive samplers will be distributed by IVL, Sweden (s. address below). For each four-week period, **two passive samplers** will be exposed to ambient air **simultaneously**. The passive samplers will arrive in small capped plastic containers sealed in a plastic bag. The containers must only be opened shortly before the start of the passive sampling/exposure, i.e. on the site. **Don't open the container until the supporting fixture (see below) is in place and sampling is ready to start!** To protect the samplers from rain, the initial delivery of the passive samplers will contain one metal disc for each site with tool holders mounted underneath to support the samplers. The measurement

technique relies on good air ventilation around the sampler. Hence, the samplers should be exposed at a height of three meters above ground, which requires a post/pole or a horizontal mounting built from a wall (see Figure 2).

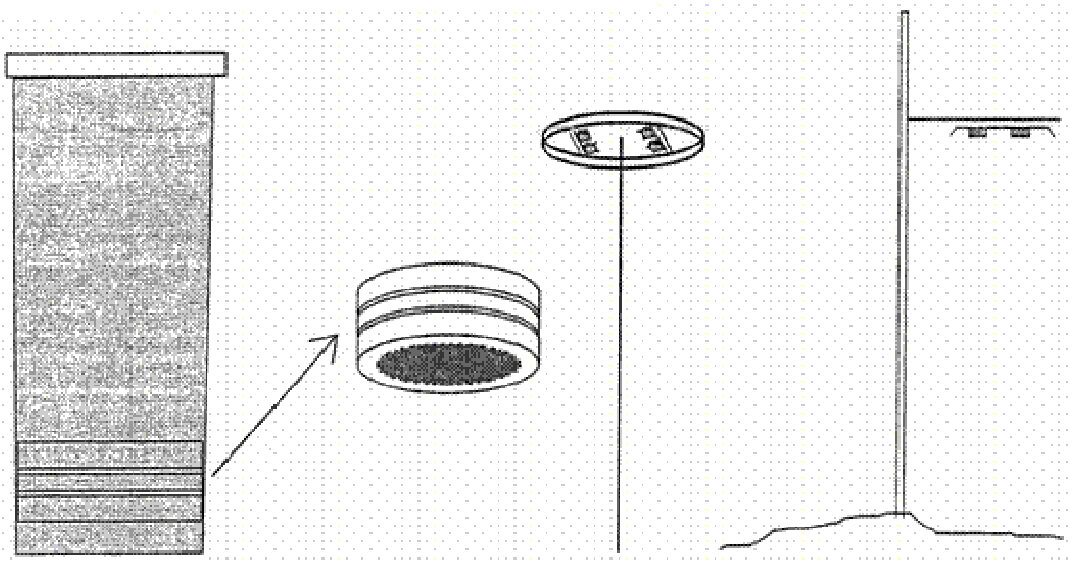

Figure 2 Set-up of passive sampling system (courtesy of IVL)

Step-by-step sampling instructions

a) Carefully remove the two samplers from the container and fix firmly to the tool holder with the grey net mesh pointing downwards. Avoid touching the net. The samplers are damaged if the net is pressed into the sampler!
b) Note date, start time and sampler location on the field protocol. Thoroughly check which samplers (number) were used in a certain time period and at a certain site.
c) At the end of the exposure period (**four-weeks**), loosen the two samplers carefully and place it in the corresponding plastic container, seal it with a cap, place the container in the plastic bag and seal the plastic bag.
d) Note the date and stop time on the field protocol.
e) Return the samplers to IVL's laboratory in Gothenburg, Sweden, preferably in a cushioned envelope. Mailing address:

IVL Swedish Environmental Research Institute Ltd.
Laboratoriet
Box 5302
S 400 14 Göteborg
Sweden

IVL will contact every participant via email once the samples have been analyzed, which might take four to eight weeks. We would like to ask you to forward their emails to Patrick Büker (pb25@york.ac.uk) in order to pool all data at one place.

Regarding delayed deliveries or other sampling problems which can arise, don't hesitate to contact one of the following persons via telephone (++46-31-7256200, IVL reception) or via fax (++46-31-7256290): Sari Honkala, Karin Sjöberg, Eva Brorström-Lundén or Tina Skårman. Alternatively, you may also contact Patrick Büker at SEI-York (pb25@york.ac.uk, ++44-1904-432890).
More detailed information on passive samplers are summarised in Appendix 1.

3. RECORDING OF METEOROLOGICAL DATA

To aid the interpretation of the biomonitoring study results, the experimental sites should either be located close to meteorological measurement stations or use micro-met loggers. All sites that are not closely located to meteorological stations will be provided with one Tinytag® data logger which records temperature and relative humidity according to defined time-steps. This time-step should be set to **30 minutes** at all sites.

The logger will come with i) a users' guide (a brief users' guide can also be found in Annex 2 of this protocol), ii) a software installation CD-ROM including a software activation code and iii) an inductive pad to download data to a computer. More detailed information on Tinytag data loggers are provided in Appendix 1.

If any problems occur concerning the installation of the software, the performance of the data loggers, the download of the data etc., please don't hesitate to get in touch with Patrick Büker at SEI-York (pb25@york.ac.uk, ++44-1904-432890).

The microloggers for temperature and relative humidity should be protected against direct sunlight in order to avoid overestimation of temperature and VPD. A simple self-ventilating radiation shield, which can be made of a piece of plastic pipe with approximately 10 cm diameter, will suffice. The Tinytag hangs in the lower part of a tube (see Figure 3), with the upper part of the tube being black and the lower part being either white or protected by a reflective cover (e.g. aluminium foil). The air will warm in the upper black part of the tube and rise, which will enable new air coming in from below. Alternatively, use a Stevenson screen if available or a simple arrangement with a shield made of aluminium foil through which air can pass, but within which the Tinytag is protected against direct sunlight.

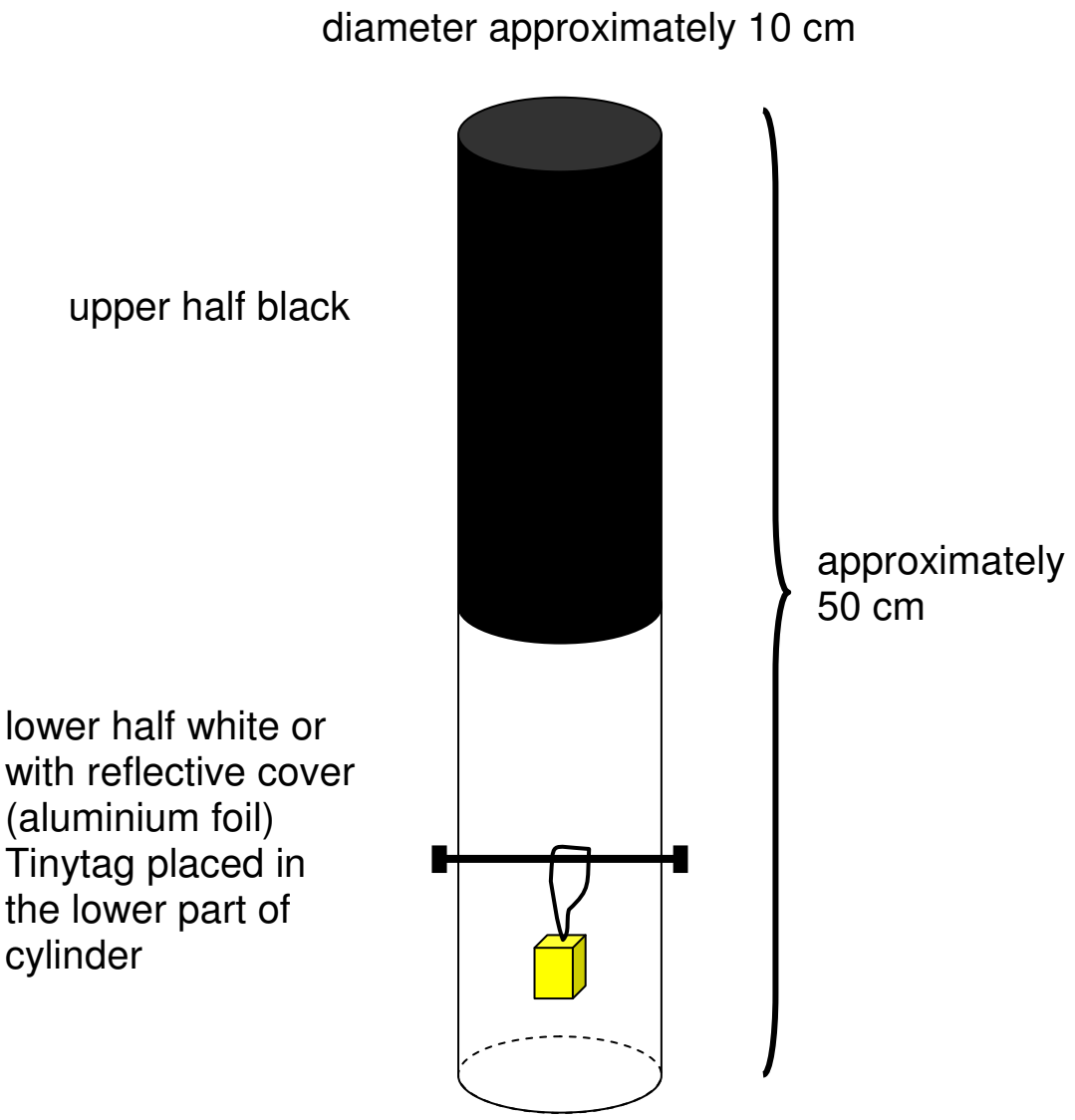

Figure 3 Example of radiation shield to protect Tinytag from direct sunlight (courtesy of Jenny Sundberg)

Since the project budget doesn't allow to provide sites with radiation measuring devices, we would like to encourage you to – if the right equipment is available – measure radiation using already existing instruments. Furthermore, if any of the following parameters are available from a near-by climate monitoring facility, then please add this information to the Excel-spreadsheet described below:

Physical climate
Air temperature (°C)
Relative humidity (%) or vapour pressure deficit (kPa)
Global radiation (Wm^{-2})
Rainfall: daily record of amount of rainfall (mm).
Wind speed ($m\ s^{-1}$) and direction (°)

Pollution Climate
Particulate Matter (PM2.5, PM 10)
Sulphur dioxide (SO_2)
Nitrogen oxides (NOx)

Ideally, the above listed parameters should be measured at 1 m height, although data from other measurement heights are acceptable, too.

All meteorological and pollution data should be collated in an Excel spreadsheet as described in Section 4.5.

4. OZONE BIOMONITORING USING WHITE CLOVER

4.1. Experimental aims and outline of experiment

- To assess the biological impacts of increased ozone concentrations on plants in South Asia
- To identify areas in South Asia where the ozone concentrations are sufficient to induce ozone-specific injury on ozone-sensitive plants (e.g. the NCS-white clover clone)
- To determine the frequency of ozone injury and to examine temporal trends of ozone injury development
- To evaluate the results of a model-based provisional risk assessment

Approx. 25 cuttings of ozone-sensitive white clover (*Trifolium repens* cv. Regal, NC-S genotype) and 25 cuttings of ozone-resistant white clover (*Trifolium repens* cv. Regal, NC-R genotype) will be distributed to participants by either CEH Bangor, U.K., or by the Veterinary and Agrochemical Research Centre, Tervuren, Belgium (the coordination team of this experiment will let participants know whether they will receive their cuttings from the U.K. or Belgium). The cuttings are transplanted into small pots, before growing them for 28 days in a greenhouse. Afterwards, the clover plants are transplanted into bigger pots (one plant per pot), before subsequently exposing them to ambient air at the selected sites (day 1 of the experiment).

Plant leaves of both clover genotypes are checked for visible ozone injury (and other leaf damage, e.g. by insects and fungi) on a weekly basis throughout the entire growing season using standardised assessment criteria.

After 28 days, the first harvest of the clover plants is performed (day 28). Three to four harvests follow at 28 day-intervals, i.e. on day 56, 84, 112 and possibly 140. At each harvest, the plants are cut back to 7 cm above the soil surface after having been assessed for visible ozone injury. The harvested material (biomass) will be dried and weighed.

Leaf injury, biomass of the cuttings, climate and ozone data are sent to the coordination team (contact details see below) of the experiment at the end of the growing season using standardised spreadsheets.

Figure 4 Experimental set-up of Potchefstroom site in 2005/06

4.2. Experimental requirements

Greenhouse: For establishing clover cuttings prior to transplanting outdoors.

Experimental Plot: The site should be situated in an open field in good distance from any pollution point sources (smelters, factories etc.) and at least 200 m from main roads and 50 m from (larger) buildings. The plot should be fenced to prevent birds and small mammals from eating the clover. The immediate surroundings of the pots should be kept free from tall growing plants (trees, bushes, tall grasses) to prevent eddy formulation from disturbed wind fields and overshadowing (see Figure 4). However, short vegetation is appreciated to prevent excessive dust formation and mud-splashes on the clover which might affect the biomass weight. The water supply should be secured.

Pots and wicks: Use 15 litre volume pots with a surface diameter of approximately 30 cm.
This pot size is needed because the clover type used grows by 'trailing stems' (stolons) which root, therefore a substantial (lot of) soil surface is required. Also, the 15 litre volume pots allow 5+ months of unlimited growth (i.e. no limiting effects of the pot wall). Each pot should have access to a water reservoir as described below (Section 4.3.2) Fibreglass wick material is supplied by our project partners from CEH Bangor (U.K.).

Soil mixture and fertilizer: A soil mixture likely to work well is local soil, sand and vermiculite in the ratio 1:1:1. It is essential that participants use a slow release fertilizer such as eight month slow release 'Plantacote pluss 8M' (14N:8P:15K (+ 2 MgO)) or products with a similar N:P:K composition. Approx. 60 g of fertilizers are required per pot.

Plant Material: Cuttings of the NC-S (ozone-sensitive) and NC-R (ozone–resistant) white clover genotypes will be supplied by either CEH Bangor (U.K.) or VAR (Belgium). Please inform the biomonitoring coordination team of the exact postal address whereto the cuttings should be sent. A plant sanitary certificate will ensure that the sent cuttings are pest free (no viruses, no insects etc.).
Please note that it is mandatory for all participants to acquire a plant import permit for fresh plant material in order to be able to receive the cuttings! This permit should be applied for way in advance of the start of the experiment to prevent any delays at the beginning of the growing season.

Monitoring equipment: Ideally, participants should have access to pollution and climate data monitored at or close to the experimental site. Hourly mean data for ozone, temperature, humidity and solar radiation are the most useful. However, where such data are not available, passive samplers will be supplied by our project partners from IVL, Gothenburg (Sweden) (cf. chapter 2).

4.3. Setting up and maintaining the experiment

4.3.1. Receiving and establishing the clover biotypes

Distribution of cuttings
Cuttings of the NC-S (ozone-sensitive) and NC-R (ozone-resistant) genotypes of white clover (*Trifolium repens*) will be supplied via express courier service by either:

Mrs. Felicity Hayes
ICP Vegetation Coordination Centre
Centre for Ecology and Hydrology
Orton Building, Deiniol Road
Bangor, Gwynedd LL57 2UP
UK
Tel: ++44 1248 370045
Fax: ++44 1248 355365
Email: fhay@ceh.ac.uk

or

Dr. Karine Vandermeiren
Veterinary and Agrochemical
Research Centre
Leuvenstesteenweg 13
Tervuren 3080
Belgium
Tel: ++ 32 2 769 22 33
Fax: ++ 32 2 769 23 05
Email: kavan@var.fgov.be

All participants will be informed by the experiment coordinators from where the cuttings will be sent (U.K. or Belgium) and of their estimated arrival time. If you are unable to plant the cuttings immediately, keep them refrigerated (but not frozen). Plant them as soon as possible in a greenhouse. The clover cuttings should be grown in 1 litre pots in the glasshouse for the first 28 days after planting as described below.

Planting procedure
The clover cuttings will be from virus-free stock, approximately 7 to 10 cm long, with 3 to 4 leaf nodes. Remove all but the youngest leaf by cutting (do not tear off).

Fill 1-litre pots with the locally selected soil mixture mentioned in section 4.2. Before planting the cuttings, water the pots thoroughly to ensure the soil mix is fully wetted.

Use a pencil (or similar item) to make a hole in the medium. Rooting is better if this is at an almost horizontal angle, rather than vertical. Place the cutting in the hole so that about 6 mm of the cutting (including apical meristem (growing tip)) remains above the surface. Firm the medium around the cutting and water-in. Label the pot to identify the genotype and replicate number.

Maintenance of the establishing cuttings

Rooting will begin immediately under reasonable conditions in the glasshouse chamber. However, cuttings will suffer if the growth medium is too dry or too moist. They will need protection from dry, hot conditions (high sunlight levels), especially during the first week. If such conditions are anticipated, place the cuttings under a plant bench or in another area protected against too strong solar radiation. High relative humidity is important. However, be careful to avoid over-watering, particularly during the first 7-10 days as this can prevent the plants from becoming established. After this, over-watering may still increase the susceptibility of the biotypes to fungi.

Keep aphids and other insects off the plants to avoid the spread of viruses and diseases. At 21 days after planting, fertilise each plant with 150 ml of a water solution containing 1 g/litre of Peters 5-11-26 (N-P-K) or close equivalent nutrient solution.

4.3.2. Transplanting

Twenty-eight days after planting the cuttings in the greenhouse they are ready to be transplanted to their final position in the experimental plot. Please note preparations for transplanting start two days before the plants are actually transplanted.
The minimum number of replicate plants to be placed at the experimental site is 20 per genotype. However, it is advisable to grow more plants in case some have to be discounted because of e.g. insect damage.

The established clover plants are transplanted into 15-litre pots (1 plant per pot) containing the chosen soil mix (see above), using the pot filling protocol described below. This will require 60g of a slow-release fertilizer (see chapter 4.2) and four 60 cm wicks and one 120 cm wick per pot.

Pot-filling procedure

Safety Information: Strong gloves, a laboratory coat, eye protection and a dust mask should be worn at all times when handling the fibreglass wicks.

Two days before planting
Make five holes in the bottom of each pot, four near the edge and one in the centre as indicated in Figure 5. Pre-cut the fibreglass wicks supplied into one 120 cm length and four 60 cm lengths per pot, and soak overnight in a bucket of water.

One day before planting
Fill the pots with the soil mix, inserting the wicks and applying the fertiliser using the following procedure:

1. Place the wicks inside the pot as indicated in Figure 5 with 20 cm extending through each drainage hole and outside the bottom of the pot. 60 cm wicks should be placed through the outer holes and the 120 cm wick should be placed through the central hole.

2. Place approximately 6-7 cm depth of soil mix into the pot and pat down firmly. Add 12 g of slow release fertilizer uniformly to the surface using a scoop calibrated to 12 g. Lay one of the 60 cm wicks clockwise in a partial circle approximately 2.5 cm in from the pot perimeter (Figure 6).

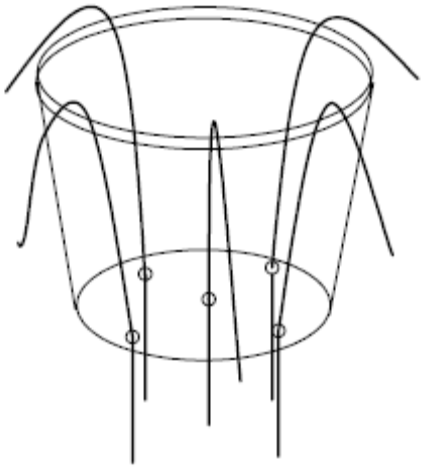

Figure 5 Placing the wicks in the pots.

3. Repeat step 2 using the adjacent wick (clockwise) and including a further 12 g of fertilizer as before.

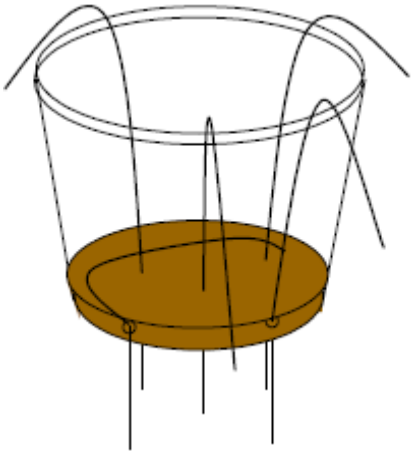

Figure 6 Wind the first wick around in a clockwise direction.

4. Repeat step 3 twice.

5. Repeat step 3 for the remaining central wick, and fill with further soil mix until close to the top of the pot, taking care to ensure that the last wick cannot dry out by being too close to the surface.

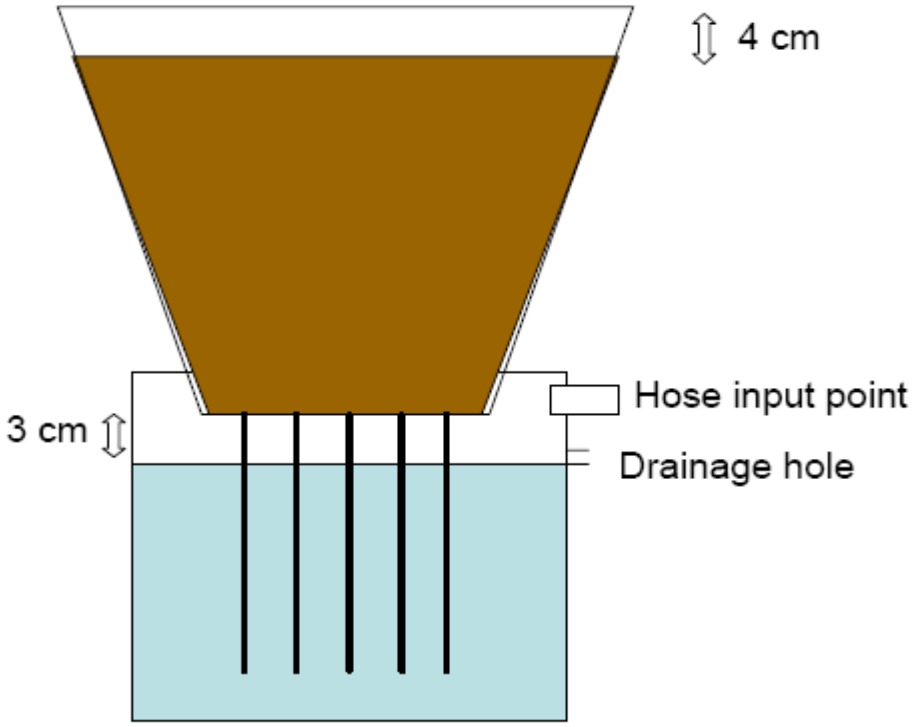

Figure 7 The pot and water reservoir system.

6. Place the pots into the filled water reservoir and water the pots thoroughly from above, top up with soil mix as necessary to make the soil surface 4 cm below the rim of the pot. The distance between the pot base and the water level should be approximately 3 cm; ensure that the pot base is not immersed in water. Drainage holes in the reservoir at this height would prevent this happening (Figure 7). The pots should be placed 0.5 m apart within rows with 1 m between rows.

7. In warm/hot climates, it is important to reduce over-heating by using white-coloured pots.

Day of planting
Use a 1 litre pot (same as the clover is growing in) to make a 'template' hole in the middle of each 15 litre pot placed on the field site the day before. Remove very carefully a 28 day old plant from its pot and place it into the 'template' hole. Firm the medium around the transplant and water thoroughly. Take care when transplanting the clover plants not to damage the shoots and roots. Standardise the surface of the soil mixture at 4 cm below the top of pot for all pots (after saturation watering). The NC-S and NC-R plants should be arranged alternately as shown in Figure 8.

S	R	S	R	S	R	S	R	S	R
R	S	R	S	R	S	R	S	R	S
S	R	S	R	S	R	S	R	S	R
R	S	R	S	R	S	R	S	R	S

Figure 8 Suggested experimental design of clover biomonitoring experiment with 20 NC-S (S) and 20 NC-R (R) clover plants

4.3.3. Establishing the plant at the experimental site and first harvest

Day 1 of the experiment is the day when the clover plants are placed outdoors at the experimental plot and is thus the day of transplanting the clover into the 15 litre pots. The plants are then given 28 days to establish in the 15 litre pots before the first harvest is performed, as described in Section 4.4.3. A first visible injury assessment according to the standards described in Section 4.4.2 is made during the second week of exposure and after that regularly on a weekly basis.

4.3.4. Care of clover plants

Watering:
The clover plants are watered via the wicks that project into the water reservoirs and natural rainfall. The reservoirs should be checked daily, especially during the last week prior to cutting back to ensure the plants are watered sufficiently. Also ensure that the media is not holding too much water so that it is waterlogged. We advise participants to build a watering system which directly connects each pot with the water supply (tap), by using tubes/pipes. This simplifies and accelerates the watering procedure!

Weeding:
Remove weeds from the pots and around the pots as necessary.

Damaged plants:
Remove any pots from the experimental plot if their clover plants have become severely damaged by anything other than ozone pollution. Do not continue to harvest these.

Insect Pests
Careful observation for insect pests is required. For example, aphids sometimes hide below stolons coiled around the inside of the pot rim. A suggested insecticide is Avid (Manufactured by Syngenta) for control of aphids, thrips, leaf miners and red spider mites. If the use of insecticides is necessary, please take notes of the product's name, chemical composition and dosage as well as the time when it was applied. Use of any pesticides should be made with consideration of prescribed safety rules.

Viruses
Virus infection normally appears as large, whitish-yellowish round spots on the upper leaf side. Remove any affected plants from the site.

Fungal infections
If the plants are infected by a fungal pathogen, fungicides can be used but please ensure that the chosen chemical will not have an interaction with ozone. For example, it is believed that benomyl and triadimefon offer some protection against ozone, and thus these and chemically related pesticides should not be used. Leaf Spot fungi can be a problem when there is a lot of rain during the growing season and Chlorothalonil (Bravo) is suggested for control. Systhane can be used to control mildew. Neither of these fungicides are thought to alter the response of the clover to ozone. Use of any pesticides should be made with consideration of prescribed safety rules.

Herbivores
Birds, rabbits and other rodents as well as possibly larger mammals feed on clover. A rabbit exclusion fence and bird netting is a requirement where these risks exist. For slug control use slug pellets (metaldehyde) or commercial biological control methods, e.g. 'nemaslug' (nematodes).

Advice:
Advice on any aspect of the experimental work can be sought from the Coordination Centre by emailing Patrick Büker (pb25@york.ac.uk). If requesting help with identifying symptoms of any type, please send photos with your email.

4.4. Assessments

4.4.1. Identifying visible ozone injury on white clover

The photographs presented in Figure 9 provide examples of ozone injury on white clover. These injury symptoms appear first on the upper leaf surface as fine pale yellow specks. Ozone injury is classed as present when 20% of the plants have one or more injured leaves.
Please note that injury caused by red spider is similar in appearance but is accompanied by webbing and the presence of minute insects (c. 1 mm diameter) on the lower surface of the leaf.

Figure 9 Examples of typical ozone injury on white clover

4.4.2. Weekly injury assessments

The following assessments should be made on the same day per week and prior to cutting back at the 28d harvests. The assessment should be made by the same two persons throughout the growing period. If possible, more than one observation of the plants per week should be made so that the date of first occurrence of ozone injury within each 28d period can be noted.

Assess each plant for ozone injury every week by indicating the percentage of injured leaves using the following scale. Please note that only fully developed leaves should be taken into consideration for this assessment.

Injury scoring system:

0: no injury
1: very slight injury; occurrence of the first symptoms
2: slight injury, 1-5 % of the leaves with slight injury
3: moderate injury, 5-25 % of the leaves with injury
4: heavy injury, 25-50 % of the leaves injured
5: very heavy injury, 50-90 % of the leaves injured
6: total injury, 90 –100 % of leaves are injured

Grade each plant as either healthy (H) or abnormal, with the cause of the abnormality graded as 1 (slight), 2 (moderate), or 3 (severe) using the following key:

O Ozone
S Stunted
D Diseased
I Insect damage
Sl Slug damage
A Animal (rabbits, deer, birds etc.)
V Virus

If the clover plants are damaged by anything other than ozone pollution, please indicate on the recording form whether you think the data obtained at this harvest should be used.

4.4.3. 28 day harvest

The cultivar of white clover used in this experiment grows rapidly. The plants need to be cut back every 28 days to ensure the development of new leaves for exposure to ambient ozone and injury assessment.

Before the harvest, if possible take (digital) photographs of the plants to give an indication of overall health and plant size.

Harvest procedure
First of all, please count the flower heads before starting the harvest. For the harvest, use a guide stick with two lines: one line to indicate the soil surface and the second 7 cm above the soil surface. Use long-blade garden shears to harvest the forage (leaves, flowers and stolons) at a height of 7 cm above the soil surface. Also, harvest forage (runners) that have grown outside the pot perimeter. Remove any injured leaves which remain after cutting so that these cannot be mistakenly identified as having new injury at a subsequent observation (do not include these leaves in any biomass determination). Dip the shears into 10% Clorox solution between pot harvests to prevent virus spread from plant to plant (use manufacturers recommended safety precautions).

Put all the plant material from each pot into a labelled paper bag, dry to constant weight (ideally at approx. 80℃ for at least 24 hours in an electric oven) and record the weight using a precision scale immediately after having cooled the paper bags down to room temperature preferably using an exsiccator or any other drying apparatus equipped with a desiccant such as silica-gel (samples not cooled down will show a too low weight due to thermal buoyancy). This is important information for quality assurance checks on the data.

Repeat this harvest procedure on at least three dates at 28 day intervals after the first cutback, i.e. on day 56, 84, 112 and possibly 140 of the experiment.

We would like to strongly encourage people to archive the clover biomass samples in order to be able to carry out additional analyses (e.g. leaf nitrogen content, heavy metal content) at some point in the future.

4.5. Data collection

Pollutant and climate data:
At the end of the experiment, we would kindly like to ask you to read out all meteorological data and create an Excel-file in the following format:

Country:			
Site:			
Geographical coordinates of site:			
Person in charge:			
Email address:			
Phone number:			
Fax number:			
Contact address:			
Comments (e.g. on missing data):			
Date	**Time**	**Temperature (℃)**	**Relative humidity (%)**
01/11/06	11:00		
01/11/06	11:30		
01/11/06	12:00		

The file should have a separate row for each hour of data, i.e. the spreadsheet should have the parameters temperature and relative humidity (plus any additional parameters recorded on sites) along the top, and day and hour in separate columns down the left-hand side. Missing data values should be represented by " * ".
Please also list the four-weekly mean ozone concentration data as recorded by the passive samplers and analyzed by IVL (see Section 2) in a separate worksheet of the same Excel file.

This file should then be sent by email to both Patrick Büker (pb25@york.ac.uk), where the data will be pooled and analyzed according to agreed standards. A receipt will be sent for any data received.

Plant data:
The injury assessment and biomass data should also be sent by email to Patrick Büker (pb25@york.ac.uk) on spreadsheets provided at the start of the growing season.

5. References

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Agawal, M., Singh, B., Agawal, S.B., Bell, J.N.B, Marshall, F., 2006. The effect of air pollution on yield and quality of mung bean grown in peri-urban areas of Varanasi. Water Air and Soil Pollution 169, 239-254.

Fuhrer, J., Skärby, L., Ashmore, M.R., 1997. Critical levels for ozone effects on vegetation in Europe. Environmental Pollution 97, 91-106.

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Mills, G. (Ed.), 2004. Mapping critical levels for vegetation, In: UNECE Convention on Long-range Transboundary Air Pollution. Manual on Methodologies and Criteria for Mapping Critical Loads and Levels and Air Pollution Effects, Risks and Trends. Constantly updated version available at: <www.oekodata.com/icpmapping>.

Mills, G., Hayes, F., Williams, P. and Harmens, H. (2005). ICP Vegetation experimental protocol for monitoring the incidences of ozone injury on vegetation. Natural Environment Research Council, 20pp.

Tingey, D.T., Olysk, D.M., Herstrom, A.A., Lee, E.H., 1993. Effects of ozone on crops. In: McKee, D.J. (Ed.), Tropospheric ozone: Human health and agricultural impacts. Lewis Publishers, Boca Raton, U.S.A., pp175-206.

van Tienhoven, A.M., Zunckel, M., Emberson, L.D., Koosailee, A., Otter, L., 2006. Preliminary assessment of risk of ozone impacts to maize (*Zea mays*) in southern Africa. Environmental Pollution 140, 220-230.

Appendix 1

The use of passive diffusive samplers and Tinytags

Jenny Sundberg & Håkan Pleijel

PASSIVE SAMPLERS

Why use passive diffusive samplers?

The main advantages include:

- It is a relatively cheap, easy to handle and reliable way of measuring
- The samplers are small, have a light weight and are silent
- No electricity is needed
- Can be used in many environments (outdoor-indoor, rural-urban, arctic-tropical)
- No field calibration is needed
- Due to this the location of the measurement site can be very geographically flexible. The samplers are good to use for regional mapping or long-term monitoring.

The key disadvantage is:

- Low time resolution (usually at least 1 week)

How do the samplers work?

During measurement the gas molecules diffuse into the sampler through the grey net. The net acts as a mechanical protection against particles. Inside the sampler the gas molecules are collected on an impregnated filter or an adsorbent material. Good ventilation of air around the sampler is required for the technique to work. After analysis an average concentration of the air pollutant during the measurement period is received.

How to use them?

The samplers are kept in plastic containers to which the cap fits tight. The containers with the samplers are placed in sealed plastic bags before and after exposure. If possible it is good if the samplers are stored in a fridge if not used immediately.

When measuring outdoors some kind of protection from rain is needed. A metal disc with tool holders mounted underneath will be provided by IVL. The disc can be placed either on a pole, out from a wall or similar. The ozone samplers should be placed about three to four meters above ground. The measurement height must be specified. It is good to describe the measurement site (or take some photos). The location in the landscape can influence the results, since for example a hilltop site is more exposed than a valley site and therefore experience higher ozone concentrations.

Do not take out the sampler from the container until it is time to start measuring at the site. The exposure starts as soon as the cap is taken off the container. Carefully take the sampler out of the container without touching the grey net, which easily can be damaged. Grip the edges. Attach the sampler underneath the metal disc with the help of the tool

holders with the grey net downwards. Enter date, starting time and name of measurement site in the field protocol enclosed in the plastic bag.

How long the measurement period should be depends on the concentration of the air pollutant at the site and the time resolution wanted. The sampler needs to be exposed long enough to pass the lower detection limit but not so long it passes the upper detection limit. A measurement period of approximately 1-4 weeks is usually good. The measurement period will be four weeks for the RAPIDC biomonitoring campaign. At least two replicates are strongly recommended.

When the measurement period is over, carefully take down the sampler and put it back into the container and close the cap tightly. Enter the date and end time in the field protocol. Any special remarks or observations should also be noted. Put the container and field protocol back into the plastic bag and seal it. Send the samplers to the laboratory so they can be analyzed. The average concentration of the air pollutant during the period of interest will be received.

What can go wrong?

- ✓ Do not open the container with the sampler until it is time to put it up. The exposure starts as soon as the cap is taken off. If the container is opened and immediately closed again the sampler will not be destroyed.
- ✓ Do not touch the grey net. It can easily be damaged. Grip the edges of the sampler.
- ✓ Place the sampler with the grey net downwards when attaching it underneath the metal disc; otherwise there will be no exposure and no result.
- ✓ Make sure the sampler is well attached to the metal disc with the tool holders so it won't fall down.
- ✓ Remember to note the start and end times of exposure as well as name of measurement location in the field protocol; otherwise the sampler will not be analysed at IVL.
- ✓ Put up some information and a "please do not touch"-note close to the sampler on the measurement site and a phone number where more information can be obtained, to avoid damage caused by curious people.
- ✓ To avoid vandalism, try to choose a measurement site that is not too exposed to people not informed about the measurements.
- ✓ Make sure the sampler is protected from rain – the metal disc should be horisontal.
- ✓ Make sure the cap is tightly closed after the measurement period is over and the sampler is put back in the container.

TINYTAG

Why use Tinytags?

The amount of an air pollutant at a site is determined by the nature of the relevant emissions and the state of the atmosphere. Meteorological characteristics such as the humidity, air temperature, intensity of solar radiation, wind, etc control the dispersion, transformation and eventual removal of the air pollutant after release.

The most important effects of air pollutants on plants are related to the uptake of the pollutant rather than to the concentration in the ambient air. The pollutant must enter the leaf through the stomatal pores, where the gas exchange between the plant and the surrounding air takes place. The degree of stomatal opening is regulated by the micro-scale climate, for example temperature, air humidity and light. It is therefore important to know how the micro- and local-scale climate varies together with air pollution concentration. If for example the air humidity is very high the stomata will be open to a larger extent and a lower ozone concentration could give rise to the same effects as a higher concentration in a drier micro environment. The stomata respond to the vapour pressure deficit (VPD) in the air. VPD is the difference between the amount of moisture in the air and how much moisture the air can hold when it is saturated. With increased shortage of moisture in the air, the plants close the stomata in order not to lose water. How much moisture the air can hold depends on temperature. Warm air can hold more moisture than cold. Therefore VPD depends on both temperature and relative humidity.

Since exposure and dose of air pollution depends on local climatological characteristics, it is necessary to combine the information about both climatology and the concentration of the air pollutant to assess the risk for effects on vegetation from air pollution. An understanding of representative measurement sites in relation to local climate is important

A relatively cheap and simple way to measure air temperature and relative humidity is to use small, robust Tinytag loggers/sensors. They do not require electricity (except for batteries which last long) and therefore the measurement site location is just as flexible as for the passive diffusive samplers.

How do the Tinytags work?

The temperature is measured by a thermistor while the relative humidity is measured with an externally mounted capacitive sensor. To be able to use the Tinytag you also need a software installed on your computer and a download cable.

How to use them?

It is very important to use a radiation shield when measuring temperature and relative humidity such as with the Tinytags. If the sunlight falls directly on the Tinytag it will warm up and of course measure the temperature of itself which is higher than the surrounding air temperature. If using a Tinytag, it is most likely that it is not possible to use a radiation shield with forced ventilation which requires electricity. One way to solve this is to use self ventilating radiation shields. The Tinytag hangs in the lower part of a

tube, the upper part of the tube is black and the lower half has reflective cover. The air will warm in the upper black part of the tube and rise and new air comes in from below. Alternatively, use a simple arrangement with aluminium foil.

New Tinytag sensors are well calibrated. It could anyway be recommended to put up the Tinytag in connection to a meteorology station with more advanced instruments some days before and/or after the measurement period of the experiment. This gives an indication of the correctness of the Tinytag sensors, the influence of the radiation shield and also if the Tintyag measures as correct in the end of the measurement period as in the beginning.

With a laptop it is easy to collect the Tinytag data on the experimental site. In case something would happen (Tinytag stolen, out of battery, etc) it is good to have collected the Tinytag data a few times during the measurement period even though it is possible to store a large amount of data in it.

Once a year it is recommended to change the battery, seal and desiccant packs of the Tinytag. To make sure that the Tinytag measures correctly it is also recommended to calibrate it once a year. After a year it will also improve the performance of the Tinytag to gently wash the relative humidity sensor with some deionised water to make sure that salt deposits will not influence the relative humidity measurements.

It is problematic to measure relative humidity close to 100%. At very high humidity out of range values may be generated by the Tinytag which have to be removed in the data processing before further calculations.

Calculation of the saturation water vapour pressure (e_s, kPa) at a certain temperature (T, °C) according to Campbell and Norman (1998):

$$e_s = 0.611 \times \exp\left(\frac{17.502 \times T}{T + 240.97}\right) \quad \text{(eq. 1)}$$

Calculation of the vapour pressure (e_a, kPa) at a certain relative humidity (RH, %):

$$e_a = \frac{e_s \times RH}{100} \quad \text{(eq. 2)}$$

Calculation of the vapour pressure deficit (VPD, kPa):

$$VPD = e_s - e_a \quad \text{(eq. 3)}$$

What can go wrong?

- ✓ Make sure the Tinytag really started; download to see if data is stored as you intended.
- ✓ Download data several times during the measurement period to make sure as little as possible is lost in case something happens to the Tinytag.
- ✓ Make sure the battery is not too old.
- ✓ Try to minimize the risk for curious people or vandals to disturb your measurements.

- ✓ Make sure the Tinytag will not be directly exposed to rainfall; it takes a while for the relative humidity sensor to dry up even though water will not damage it.
- ✓ Remove out of range relative humidity values before forming averages over longer times.

READ MORE

http://www.ivl.se/en/
http://www.tinytag.info/

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Carmichael G. R., Ferm M., Thongboonchoo N., Woo J. H., Chan L. Y., Murano K., Viet P. H., Mossberg C., Bala R., Boonjawat J., Upatum P., Mohan M., Adhikary S. P., Shrestha A. B., Pienaar J. J., Brunke E. B., Chen T., Jie T., Guoan D., Peng L. C., Dhiharto S., Harjanto H., Jose A. M., Kimani W., Kirouane A., Lacaux J. P., Richard S., Barturen O., Cerda J. C., Athayde A., Tavares T., Cotrina J. S. and Bilici E. (2003) *Measurements of sulphur dioxide, ozone and ammonia concentrations in Asia, Africa and South America using passive samplers*. Atmospheric Environment 37: 1293-1308

Ferm M. (2001) *Validation of a diffusive sampler for ozone in workplace atmospheres according to EN838*. Proceedings from the International Conference on measuring Air Pollutants by Diffusive Sampling, Montpellier, France, 26-28 September, pp 298-203

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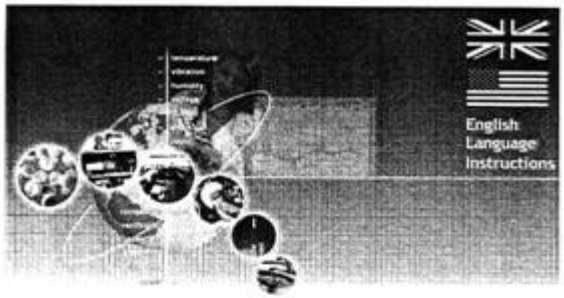
Appendix 2



Tinytag Explorer

Quick Start Guide

Tinytag

WHAT YOU NEED

- **Tinytag Explorer software installation CD-ROM**
- **Software Activation Code**
- **One or more data loggers**
- **Download connection cable or inductive pad from data logger(s) to PC**
- **External probe (for loggers requiring an external probe only)**

INSTALLING THE SOFTWARE

- Start your computer, and insert the Tinytag Explorer CDROM into the drive.
- In a few seconds, the installation process should start automatically
- At the installer welcome screen, click **Next** and follow the instructions.
- Read the **Licence Agreement**. For installation, accept the conditions. Click **Next**.
- **Select Installation Folder**: the system asks you where you want to install the program. We recommend that you accept the default and click **Next**.
- **Ready to Install**: click **Install** to proceed. Installation will take a few moments.
- Once complete, click the **Finish** button to exit. The software is now installed and once you have re-started your computer you can begin to use it.

"Nothing happened after I put the CD in"

- Open **Windows Explorer** and navigate to your CD-ROM drive. It may be labelled Compact Disk or CD-RW drive.
- Look at the files on the CD and double-click on **setup.exe** (if you cannot see it, look for **tinytag.msi**). The installation should now begin.

ACTIVATING THE SOFTWARE

- When you come to the **Activate** screen, type the code into the box provided.

Example Activation screen showing a 'dummy' code...

- Click **Confirm Code** to activate the software.

Activation code not recognised

If the code is not recognised, the system will tell you.

- Click **OK** to clear the warning screen.
- Try entering the code again, typing the letters and numbers carefully. Click **Confirm** to activate. The main screen should now appear.

Note: If you cannot successfully activate the software, please call Gemini Technical Support.

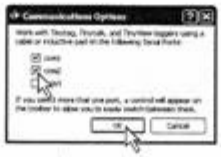
Time-limited demo trial software only

If you are trying the software before you buy it, click **Start Demo**. The system will work until the expiry date shown and then will cease to function.

SERIAL PORT SETUP

Once you have installed the software, it can detect **how many serial ports** there are on your computer.

Start the software and go to **menu > Options > Communications Options.**



Example where two serial ports are used for loggers...

- Check ☑ those port(s) to which you will connect loggers, and leave the others ☐ unchecked. The default is com1.

CONNECT LOGGER TO PC

There are two types of connection at the logger end of the cable, a conventional **cable plug** or a Tinytag **data transfer pad**. At the computer end, there is either a 9-pin D-connector or a USB connector, depending on what was specified.

Plug the D-Connector into the Computer

- If the cable has a 9-pin D-connector, plug it into a serial port on the back of your PC.
- Make sure the plug is the right way up, push it in firmly.
- Tighten the fixing screws so that the plug is secure. Do not over-tighten.

Plug USB Cable into the Computer

- Another version of the cable uses a USB connector rather than a 9-pin D-type connector.
- Plug this into a USB port on your computer.

Logger Connection by Plug

- If you have loggers that use a plug connection, note that the socket on some loggers has a 'key' for aligning the plug.
- Rotate the plug until it clicks into place, then push firmly home.

- Other loggers use a 3.5mm jack plug (resembling the kind used on headphones).
- Align the plug carefully and push it into the small socket until it clicks into place.

Logger Connection by Inductive Pad

- Just place the logger onto the inductive pad and you are ready to transfer data to and from the computer.

CONNECTION FUNCTIONS

- Use **Toolbar > drop-down** to select Com port to which the logger is connected.

Functions available

- Launch: See **Configure and Launch.**
- Stop: To stop the logger **from recording any more data.**
- Get data: See **Get Data from Logger.**
- Logger's current readings.

Failed to connect to data logger on com...

- If the system cannot 'see' the logger, check the cable connections at both ends.
- Check that the correct Com port is selected.

CONFIGURE & LAUNCH LOGGER

WARNING: Make sure you download and save data **before** you configure and launch a logger, otherwise you will **lose all previous recorded data.**

- To start the setup process, click **toolbar > Go**.

In a few seconds the **Configure and Launch** screen appears. This shows the options available for the logger. The upper part of the screen shows the list of options, configure each option in the lower part. Use **Next** / **Back** to move from step to step.

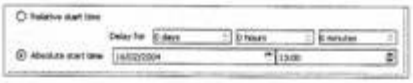
Page 1 of 6: Enter a Description

- Enter your own description for the next measurement log. This will be stored with the captured data and appear on charts etc.

- Click **Next.**

SET START TIME

Page 2 of 6: Start Options



- Tell the logger when to begin logging data.
- Click ⦿ to set a Relative Start Time. Delay = 0 means start now. OR
- Click ⦿ to set a Relative Start Time + Delay = X days, Y hrs and Z mins after you click **Launch**. OR
- Click ⦿ to set an Absolute start time and date.
- Click **Next**.

As well as the common settings shown above, some loggers have extra settings for specific purposes.

SET TRIGGERED START

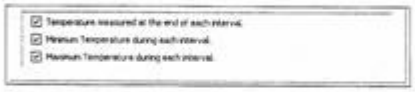
Page 2 of 6: Select triggered start. (Not all models)

Some loggers have a **magnetic switch** built in. You activate the logger by holding a magnet close to the front of it.

- Check ☑ Wait until trigger event. You can also add a delay if you wish.
- Click **Next** to continue.

SELECT MEASUREMENTS

Page 3 of 6: Measurements



Some models only have the Spot Measurement option; but if there is a choice, check ☑ whether you want Spot Reading, Minimum and/or Maximum values:

- ▷ **Spot value** = instantaneous value **measured at the end of each logging interval.**
- ▷ **Minimum** = lowest value **during each logging interval.**
- ▷ **Maximum** = highest value **during each logging interval.**

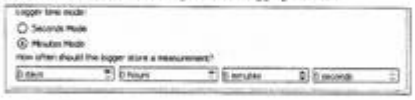
Note: If you want to **record Minimum and Maximum** readings, you must select the **Minutes Mode** in the next screen.

- Click **Next**.

SET LOGGING INTERVAL

Page 4 of 6: Logging Interval

How often do you want to take a reading? Set the logging interval.



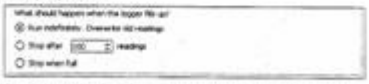
There are **two logging modes**:

- Click ⦿ **Seconds Mode** for short intervals where readings may fluctuate quickly. In this mode, you cannot take Maximum and Minimum readings, and you cannot download data while the logger is recording. OR
- Click ⦿ **Minutes Mode** for longer intervals over longer periods of time. This mode also allows you to check the logger's current reading at any time, and to take maximum and minimum readings, and to download while still running.
- Then click **Next**.

Note: You must set a **valid time interval** or you cannot launch the logger.

SET STOP OPTIONS

Page 5 of 6: Stop Options



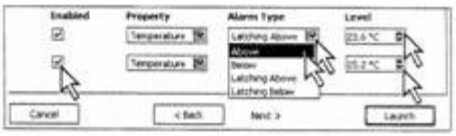
- ▷ Run indefinitely - **this fills the memory to the end and then starts writing again at the beginning. This will** overwrite old readings **as it goes.**
- ▷ Stop after N readings - **takes N measurements only before stopping. Select the number required.**
- ▷ Stop when full - **takes readings until the** memory is full **and then stops.**

- Click **Next**.

SET ALARM INDICATORS

Page 6 of 6: Alarm Options. (Not all models)

This sets a **visible alarm indicator** if a logger detects a reading **above or below preset thresholds**.



- Enabled: Check ☑ to enable each alarm as required.

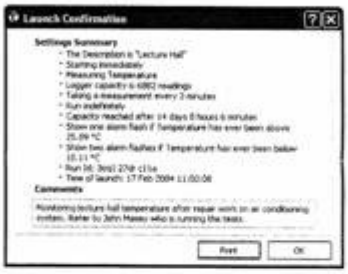
LAUNCH AND PRINT SUMMARY

⚠ **WARNING**: Once you **launch the logger**, you will **lose all previous recorded data**. To download the data first, see **Get Data from Logger**.

- Click **Launch** [Launch] to save the settings to the logger and start it operating with the new settings.

The system confirms the launch and displays the **Settings Summary**.

- You can add Comments if you wish.



Example environment temperature logging in a University Lecture Hall...

Note: The Comments only appear on the printout - they are not stored in the logger.

- Click **Print** to print this information to your computer's printer, then **OK** to finish.

Note: the **Run Id** is created by the software as a unique number for that logging run, except when you use a triggered start. It will appear in the download information.

- You can now unplug the logger and set it in place for recording data.

If the logger has LED indicators, one of them will flash every few seconds when it is recording. (The flash interval depends on the logger settings.)

GET DATA FROM LOGGER

Note: This does **not** clear the stored data, provided you have not launched another recording session - you can download it again if required.

- Connect the logger to the computer, and select the correct Com port (if you have more than one).
- Click **Toolbar > Get Data from logger**.

If a logger is set to **Seconds mode**, it **cannot download** while it is still running. The system will ask you to confirm that it should **stop the recording process** first.

- Click **Yes** to proceed.

Wait a few seconds while the computer downloads the data.

If there is a lot of data, this could take a minute or two. When it has finished, you will see the **Graph** (or chart) on the screen.

SAVE DATA

Note: If the data looks good quality, we recommend that you **Save Data** to a file before you go any further.

- Click on the Save icon.
- Navigate to where you save your data files.
- Enter a suitable file name and click Save.

FURTHER INFORMATION

For further information, refer to the Online Help Guide installed with Tinytag Explorer.

- Start Tinytag Explorer, and then go to **menu > Help > Contents**.

Alternate language versions of manuals and guides are available the Gemini website at: **www.geminidataloggers.com/manuals**

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9600-0006 Iss 1.1 2005-01-07