United Nations Economic Commission for Europe

Convention on Long-range Transboundary Air Pollution

International Co-operative Programme on Assessment and Monitoring of Air Pollution Effects on Forests (ICP Forests)

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# MANUAL

on

methods and criteria for harmonized sampling, assessment, monitoring and analysis of the effects of air pollution on forests

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Part VIII

## Assessment of Ozone Injury

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# 1. Introduction

The essential basis for choosing ozone ($O_3$) induced visible injury is that many plant species respond to ambient levels of ozone pollution with distinct visible foliar symptoms that can be diagnosed in the field.

The results attributed to LEVEL II sites will be documented in maps covering Europe, characterizing areas of increased ozone risk for European forest ecosystems. However, the development of ozone-induced injury is inter- and intra-species specific, and depends apart from local ambient ozone concentrations on other environmental such as biotic and climatic factors. Due to the complex nature of the diagnosis and the given restrictions of the investment, results from the tree and vegetation assessment should be considered as semi quantitative.

Ozone pollution, unlike fluoride or sulfur dioxide pollution leaves no elemental residue that can be detected by analytical techniques. Therefore, ozone-induced visible injury on needles and leaves is the only easily detectable evidence in the field as a result of oxidative stress, leading to a cascade of adverse physiological and morphological effects. Until now, experiments have concentrated on explaining the mechanisms leading to injury observed in experimental studies, rather than to identify and characterize the symptoms observed in the field on a regional scale. The evidence we have today strongly suggests that ozone occurs at concentrations that cause visible foliar injury to a wide range of sensitive plants. Even though ozone visible injury does not include all the possible forms of injury to trees and natural vegetation (i.e. pre-visible physiological changes, reduction in growth, etc.), observation of typical symptoms on above ground plant parts in the field – also referred to as *passive bio-indication* - has turned out to be a valuable tool for the assessment of the impact of ambient ozone exposures on sensitive species in Europe.

The assessment of ozone visible injury serves therefore as a means to estimate the potential risk for European ecosystems that are exposed to elevated ambient ozone concentrations and has to be considered in the context of ICP Forests aiming among others to document the presence of environmental drivers that may affect forest condition across Europe.

# 2. Scope and application

Many plant species respond to elevated ambient levels of tropospheric ozone ($O_3$) with distinct specific foliar symptoms. These symptoms can be diagnosed only after adequate training (see chapter 5.2). This Part of the Manual aims at providing a consistent methodology to collect high quality, harmonized, and comparable data on ozone-induced visible injury on native vegetation at the intensive Level II plots of the monitoring program (Table 1). Harmonization of procedures is essential to ensure spatial and temporal data comparability. For the injury data being accepted for the international database and evaluations, National Focal Centers and their scientific partners that are participating in the UN/ECE ICP Forests program must follow the methods and apply the manual described herein.

**Table 1**. Reference list of variables to be measured for the assessment of ozone visible injury at Level II plots

| Survey | Form | Variable | Reporting unit | Level II | Level II core | Level I |
|---|---|---|---|---|---|---|
| In-plot survey | | | | | | |
| OZ | PLL | Soil moisture | Soil moisture classes | o | o | n |
| OZ | LTF | Percentage of symptomatic, current year's leaves & needles on main tree species | Score per branch | o | o | n |
| OZ | LTF | Percentage of symptomatic, last year's needles (C+1) on main tree species | Score per branch | o | o | n |
| Off-plot survey | | | | | | |
| OZ | LSS | Symptomatic species per selected quadrate (LESS) | Species name & code | o * | m | n |
| OZ | LSS | Non-symptomatic species per selected quadrate (LESS) | Species name & code | o * | m | n |
| OZ | OTS | List of symptomatic species outside of selected quadrates (LESS-plus) | Species name & code | o * | o | n |

o = optional; m = mandatory; n= not measured
* recommended for plots where ozone concentrations are measured
In-plot survey: within Intensive Monitoring plot
Off-plot survey: LESS and LESS-plus

## 3. Objectives

The main objective of assessing ozone visible injury on a selected number of Level II plots is to assess the effect of tropospheric ozone at the sites where ozone monitoring is performed, and to contribute to an ozone risk assessment for European forest ecosystems.

Specific aims are set as follows:

Quantification of ozone injury occurrence on a selected number of Level II plots in Europe.

Detection of temporal trends on a selected number of Level II plots in Europe (significant changes within 10 years with a 95% significance level for individual plots).

## 4. Location of measurements and sampling

The assessment of ozone visible injury is to be conducted within the vicinity of the plots where ozone monitoring is carried out. Both in-plot and off-plot assessments are considered.

*In-plot assessment* is conducted within the Intensive Monitoring Plot. It is optional, for the main tree species on Intensive Monitoring Plots and has to be carried out on leaves of the upper, fully sun exposed crown, every second year.

Off-plot assessment is conducted:

Quantitatively (LESS): Since most of the intensive monitoring plots are situated in closed (shaded) forests and visible ozone injury is usually restricted to the sunlight exposed upper most crown part, a **L**ight **E**xposed **S**ampling **S**ite (LESS) has to be installed in the vicinity of the open monitoring plot with passive samplers. This site serves the monitoring of visible ozone injury at an extended number of species including, if accessible, the main tree species. The survey should be done on an annual basis.

Qualitatively (LESS-plus): A list of symptomatic species is developed. This list includes tree and shrub species that are grown i) in the quadrates that have not been selected (randomly) for the quantitative assessment within the LESS, and ii) outside of the LESS, but within a radius of 500 m. The abbreviation for this area to be investigated is called LESS-plus.

## 4.1 Sampling design

### 4.1.1 Sampling design for in-plot assessment

4.1.1.1 Criteria for species and tree selection and branch sampling

The procedure includes the selection of main tree species for symptom evaluation at each intensive monitoring plot. The ozone symptom evaluation shall comprise:

The assessment for visible ozone injury on main tree species is conducted on the leaves from the same branches where foliar analysis is carried out (see ICP Forests Manual, Part XII, Foliar Sampling and Analysis).

The samples for foliar injury are collected every second year from the upper sun exposed crown. (For further details, see ICP Forests Manual, Part XII, Foliar Sampling and Analysis)

For deciduous species (incl. broadleaf species and *Larix* spp.), current year leaves (C) and for evergreen species (incl. conifers and *Quercus ilex*), current year (C) and previous year (C+1) leaves are assessed for ozone visible injury.

### 4.1.2 Sampling design for off-plot assessment on LESS

A Light Exposed Sampling Site (LESS) is established within the vicinity of the open-field meteorological monitoring station or deposition samplers where ozone concentrations are recorded (Annex I). The aim of the assessment within the LESS is to provide estimates of ozone visible injury on the vegetation at the light exposed forest edge closest to the ozone measurement device within a maximum radius of 500 m (78.5 ha). A number of 2 x 1 m quadrates are randomly selected along the forest edge in order to assess the presence and absence of ozone visible injury on the respective species.

4.1.2.1 Sampling scheme

The suggested sampling scheme is a random sampling design as described in Annex I.

4.1.2.2 Number of replicates

The number of replicates consists of the number of randomly selected quadrates which depends on the length of the total LESS. Details on how to calculate the number of sampling units are outlined in Annex I.

## 4.2 Sampling equipment

Minimum equipment required for the assessment of ozone visible injury in the field:

A 10x hand lens for closer examination of ozone visible injury on the plant leaves

The respective plot maps and a compass to determine exact location (coordinates), exposition, and elevation of the LESS

Reference pictures to assist in symptom identification of known sensitive species: Relevant information can be found on the ICP Forests web page of the WG on Ambient Air Quality

A plant press to store the leaves

A digital camera to take pictures

Plastic bags for fresh sample materials

Field data sheets

A cooler of sufficient size to accommodate storage of voucher specimens; equipment for microscopic sampling if required.

## 4.3 Frequency of sampling

### 4.3.1 In-plot assessment

Identification and quantification of ozone visible injury for conifer- and broadleaf-species within the intensive monitoring plot shall be carried out during the periods recommended for the chemical foliar analysis and according to gathered experience within the ICP Forests framework. Otherwise, it should be carried out based on the regional phenology of the present species within the intensive monitoring plot:

For evergreen main tree species: October - February

For deciduous main tree species: July - beginning of September

An annual assessment is preferred.

### 4.3.2 Quantitative off-plot assessment (LESS)

Identification of ozone visible injury on trees, shrubs, and vines within the LESS are carried out at least once during late summer before natural leaf discoloration sets in.

### 4.3.3 Qualitative off-plot assessment (LESS-plus)

Identification of ozone visible injury on trees, shrubs, and vines are grown: i) in the quadrates that have not been selected (randomly) for the quantitative assessment within the LESS, and ii) outside of the LESS, but within a radius of 500 m.

## 4.4 Sample collection, transport and storage

Symptoms have to be documented with pictures. The pictorial collection is required for the validation of ozone visible injury observed in the field by the evaluation teams. This collection serves as national documentation.

### 4.4.1 Pictorial samples

During each annual evaluation period, *pictorial samples* in form of digital photographs should be collected of two symptomatic and two non-symptomatic leaves (preferably small branches) per symptomatic species showing ozone visible injury, visible ozone-like symptoms respectively if not confirmed yet. For each symptomatic leaf, pictures of the entire plant, and the upper and the lower leaf surface should be taken.

The following guidelines are strongly recommended for *quality assurance* and uniformity of the pictorial documentation:

Pictures should be taken under full sun light exposition or with a camera equipped with a flash.

The digital picture should have a minimum resolution of 1500 pixel per inch and be stored in JPG- or TIFF-format without any picture corrections being applied.

The leaf sample should cover at least 3/4 of the final picture area to enable proper symptom identification.

In addition, it is recommended to zoom on typical and species-specific ozone symptom characteristics such as shading effect, non-symptomatic leaf veins, age effect (entire branch/plant) etc. as described in chapters 5.1.1.1 and 5.1.1.2.

Each picture file should be labeled with the following specific code for proper long-term data management and storage: XXPPPPNNNNDDDDDDTTTTTTSS.jpg

XX – country code (ICP Forests manual, Expl. Item, forms document www.icp-forests.org/Manual.htm)

PPPP – plot number (ICP Forests manual, Expl. Item, forms document www.icp-forests.org/Manual.htm); "9" and 3 further letters for assigning a location not being a ICP Forests plot

NNNN – 3 digit tree species code followed by “1” in case of ozone damage and “0” in case of no ozone damage observed.

DDDDDD – date of image production (DayMonthYear: e.g. 140509)

TTTTTT – time of image production (HHMMSS)

Sequence number (01, 02, 03,.) to indicate which photo in a respective time.

For each symptomatic species, the above described pictures must be made available to the National Focal Centre for documentation and/or further evaluation, representing each symptomatic species listed in the final data form.

In cases of doubts and/or special interest, the ICP Forests WG on Ambient Air Quality should be contacted for support and additional investigations (see chapter 5.2).

The names of the photo files are submitted to the international data centre with form OZP (see forms document available at ICP Forests webpage).

# 5. Measurements

## 5.1 Variables measured and reporting units

For the in-plot assessment, the following variables are measured (Table 1):

Symptomatic leaves or needles, reported as frequency classes (% score of symptomatic leaves for each sampled branch per IM plot)

For the off-plot (LESS and LESS-plus) the following variables are measured (Table 1):

Lists of symptomatic and asymptomatic species per quadrate (LESS). ‘Empty’ quadrates such as a gap, skidder trail, rock, etc., where no woody species are growing are also recorded. See also chapter 6.4.

A list of symptomatic species within a radius of 500 m from the ozone sampling device (LESS-plus).

### 5.1.1 Symptom identification

The following recommendations should be followed for the scoring of visible ozone injury.

#### 5.1.1.1 Symptom identification for broadleaf species

Ozone visible injury can be identified and distinguished from symptoms caused by other biotic/abiotic factors by the following criteria (see also Annex II):

1. Visible symptoms are typically expressed as tiny purple-red, yellow or black spots (described as stipple) or sometimes as a general even discoloration, reddening or bronzing.
2. Look for ozone visible injury on fully developed, light-exposed leaves.
3. Symptoms are more severe on mid-aged and older leaves than on younger leaves. Older leaves are the first ones that develop symptoms followed by an accelerated senescence (age effect).
4. Shaded portions of two overlapping leaves do not show any visible injury (shade effect).
5. Ozone visible injury normally does not go through the leaf-tissue (exception, see point 6). Both, stippling and even discoloration, occur only between the veins (interveinal) and do not affect the veins.
6. Towards the end of the growing season, foliar symptoms may progress to leaf yellowing or premature senescence, followed by earlier leaf loss. Severely injured leaves may develop necrosis that can also be seen on the lower leaf surface towards the end of the growing season.
7. Plants growing on more humid sites are more likely to develop ozone visible ozone injury compared to plants grown on drier sites (higher $O_3$ uptake).

Examine ozone visible injury as described below, using a hand lens and the flow chart in Annex II:

Is there any stippling?

Is there any reddening and/or even discoloration?

Do the symptoms, as described above, occur on the upper leaf surface only (except during late season when visible injury becomes more severe and necrotic)?

Are the symptom expressed between the veins only and are absent on the veins and veinlets (use hand lens and hold leaf against the light)?

Are the symptoms evenly distributed?

Are the symptoms more developed on the older leaves (including leaflets, 'age effect')?

If the above questions are answered affirmatively, the symptom can be considered as ozone visible injury. Additional information on species-specific symptom expression is provided on the ICP Forests web page of the WG on Ambient Air Quality.

#### 5.1.1.2 Symptom identification for conifer species

Ozone visible injury on conifer species is expressed at the upper parts of the crown, in the upper side of branches and needles. For identification follow the criteria below:

1. Chlorotic mottling is the most common ozone-induced symptom described for conifer needles; it is the result of chronic exposure to ozone and can be described as yellow or light green areas of similar size without sharp borders between green and yellow zones. However, not all needles in a fascicle may be uniformly affected.

2. Chlorotic mottling frequently appears only in needles older than 1 year (second-year needles and older). That is, the observed symptom seems to increase with increasing needle age (age effect).
3. Chlorotic mottling is more distinct on light-exposed needle areas in comparison to shaded ones (shade effect).
4. It is easier to observe the mottling, if several needles are held close to each other, forming a "plane" of needles.

Examine ozone visible injury as described below, using a hand lens:

Is chlorotic mottling present in the current +1 and more intensively in the current +n year needles

Is the color of the mottling yellow or light green?

Is the shape of the mottling areas regular with diffuse borders?

Is the mottling evenly distributed along the entire needle, and more intense in the abaxial surface or most light exposed needle side?

If the above questions are answered affirmatively, the symptom can be considered as ozone visible injury.

Special attention has to be paid to confounding symptoms such as symptoms caused by spider mites and sucking insects. Using a hand lens helps to detect their remnants easily. Additional information about mimicking factors is provided on the ICP Forests web page of the WG on Ambient Air Quality.

### 5.1.2 Evaluation and scoring

A minimum of 3 branches per tree and 5 trees per plot are assessed. Evaluation shall be different for broadleaf and conifer species. The following protocols are suggested.

#### 5.1.2.1 Broadleaf trees (main tree species and others) within the Intensive Monitoring Plots

Once the branches are collected (see chapter 4.3.1.), all leaves per branch are examined under best light conditions and scored for occurrence of ozone visible injury (yes/no). According to the scoring system in Table 2, the percentage of symptomatic leaves per branch is estimated and scored.

**Table 2.** Scoring and definition for the percentage of symptomatic leaves on a branch with approximately 30 leaves

| Score | Frequency class (%) | Definition |
|---|---|---|
| 0 | No injury | None of the leaves are injured |
| 1 | 1 - 5 % | 1 – 5% of the leaves per branch show ozone symptoms |
| 2 | 6 - 50 % | 6 – 50% of the leaves per branch show ozone symptoms |
| 3 | 51 - 100 % | 51 – 100% of the leaves per branch show ozone symptoms |

#### 5.1.2.2 Conifer trees (main tree species and others) within the Intensive Monitoring Plots

Once the branches are collected (see chapter 4.3.1), the different needle age classes are identified. Only current year needles (C) and current + 1 year needles (C+1) are assessed. Needles have to be placed close to each other (making a "plane", at least 30 needles if available) and examined in full sunlight. The chlorotic mottling will be scored for each needle age class in percentage of total surface affected. A computer-generated simulation with ideal visible injury patterns and scores is available to assist with this (Annex III). The resulting percentages per branch and needle age are then transformed to the corresponding score (classes), according to Table 3.

**Table 3**. Scoring and scoring definition for visible ozone injury as it is expressed on the respective needle years for the collected branches of conifer species

| Score | Frequency class (%) | Definition |
|---|---|---|
| 0 | No injury | No injury present |
| 1 | 1 - 5 % | 1 – 5% of the surface is affected |
| 2 | 6 - 50 % | 6 – 50% of the surface is affected |
| 3 | 51 - 100 % | 51 – 100% of the surface is affected |

5.1.2.3 Woody species (trees, shrubs and vines) within the LESS

For the symptom assessment of small trees, shrubs, and vines within the LESS, the procedure as described in Annex I should be applied. The following data are required for each of the randomly selected quadrate (spatial sampling unit):

The scientific name and code of the relevant species with the indication whether they show symptoms or not. For special cases of 'empty' quadrates, see chapter 6.4.

When undertaking the assessment, the following guide lines apply:

The assessment is conducted on trees, shrubs, and vines;

The plant nomenclature must refer to the *Flora Europaea* species codes;

Pictures of each injured species should be collected in accordance to section 3.4.

Record soil moisture conditions within the LESS and the optional subplots according to Table 4. If conditions vary markedly across the site, make a note on the result sheets and mark it on the map.

**Table 4**. Code and definition for the classification of the soil moisture conditions within the LESS

| Code | Definition |
|---|---|
| 1 | Wet or damp (e.g., riparian zones and wet or damp areas along a stream, meadow or bottom land) |
| 2 | Moderately dry (e.g., grassland or meadow, and North or East facing slopes) |
| 3 | Very Dry (e.g., exposed rocky edges) |

5.1.2.4 Woody and herbal species within the LESS-plus

To achieve a more complete list of symptomatic species around the ozone monitoring device, in addition to the survey within the LESS, the forest edges within a radius of 500 m of the passive sampler's location can be qualitatively assessed (optional), and symptomatic species recorded. Provide both, name and species code.

## 5.2 Quality Assurance and Quality Control

The *standard for ozone visible injury assessment* is based on the pictorial atlases provided by the ICP Forests WG on Ambient Air Quality (see ICP Forests web page of the WG on Ambient Air Quality). The pictures contain species-specific symptom expressions on conifer and broadleaf species, including individual diagnostic descriptions for various species developing ozone-like symptoms, confounding symptoms, and phenologically related information.

In addition to the pictorial atlases, an *'On-Line Tool for Ozone-like Symptom Validation'* is available on the web (see ICP Forests web page of the WG on Ambient Air Quality). Where identification of visible injury is uncertain, digital pictures of unknown or confusing ozone-like symptoms can be uploaded. The pictures will be assessed by a team of independent experts from the WG on Ambient Air Quality and a report will be returned.

For additional validation, *microscopic analyses* are recommended. For sampling methods and required equipment, see Annex IV. Fresh or fixed samples for microscopy can also be sent to this expert group of the WG on Ambient Air Quality for further investigations.

For training and intercalibratioin, the WG on Ambient Air Quality organizes an international *Intercalibration Course on the Assessment of Ozone Visible Injury*, each year.

*National Focal Centres* collect information and co-ordinate national efforts, including the documentation of injuries found on new species. The National Focal Centres are responsible for their field teams to be trained in visible symptom identification, quantification of foliar injury symptoms, and sampling. Field teams are trained within their respective countries by the persons who attended the international Intercalibration Courses.

### 5.2.1 Plausibility limits

For the variable (scoring of the branches) within the In-Plot survey, the value should be between 0 and 3. For the variables (species names and codes) measured in the Off-Plots surveys, no plausibility limits can be established.

### 5.2.2 Data completeness

Data completeness requirements for the assessment of ozone visible injury will be evaluated in terms of number of assessed branches (in-plot assessment) and quadrates (off-plot, LESS assessment) that are *reported* vs. the *expected* ones. Data completeness requirements for the assessment of ozone visible injury are provided in Table 5.

**Table 5.** Data completeness requirements for the assessment of ozone visible injury

| Variable | Reporting unit | Data completeness |
|---|---|---|
| In-Plot survey | | |
| Symptomatic leaves or needles per branch and plot | Score per branch | At least 80% of the required branches scored |
| Off-Plot survey | | |
| No. of quadrates assessed (LESS) per plot | Species name & code | At least 80% of the expected number of quadrates according to the adjusted sample size |

Notes
In-plot survey: within intensive monitoring plot
Off-plot survey: LESS and LESS-plus
For definition of adjusted sample size, see Annex I, Table A-1.

### 5.2.3 Data quality objectives or tolerable limits

Data quality will be improved by means of intercalibration exercises. Typically, two types of exercises will be carried out: indoor exercises, consisting of assessing correctly (Yes or Not) ozone-induced visible injury from pictures and fresh materials, and outdoor exercises where the LESS methodology is applied. The data quality objectives for both types of exercises are outlined in Table 6.

**Table 6**. Data quality objectives (DQO) for individual expert assessing ozone visible injury

| Type of exercise | Variable | Data quality objectives |
|---|---|---|
| Photo exercise | Scoring (symptomatic or not) of several plants | ≥ 70% agreement with control |
| Fresh material exercise | Scoring (symptomatic or not) of several plants | ≥ 70% agreement with control |
| LESS survey | Number of quadrates including symptomatic plants | Control ± 2 quadrates |
| LESS survey | Number of symptomatic species per LESS | Control ± 2 species |

### 5.2.4 Data quality limits

The data for ozone visible injury are considered of sufficient quality when the following criteria in Table 7 are met:

**Table 7.** Data quality limits for the assessment of ozone visible injury

| Type of exercise | Variable | Data quality limits |
|---|---|---|
| Photo exercise | Scoring (symptomatic or not) of several plants | ≥ 70% of the individuals fulfill DQO |
| Fresh material exercise | Scoring (symptomatic or not) of several plants | ≥ 70% of the individuals fulfill DQO |
| LESS survey | Frequency of quadrates including symptomatic plants (% of forest edge vegetation area affected) | ≥ 70% of the individuals fulfill DQO |

# 6. Data handling

## 6.1 Data submission procedures and forms

All validated data must be submitted in electronic format to the central data base at the ICP Forests Programme Coordinating Centre (IPCC), using the forms that are provided by ICP Forets through the National Focal Centres as soon as possible but in the calendar year following the observations at the latest. The detailed time schedule is provided by the relevant bodies of IPCC. The person responsible for the national database should inform the National Focal Centres about the different methods of electronic data submission.

## 6.2 Data validation

Data will be checked for plausibility and completeness, based on the results from the intercalibration exercises (DQO and DQL established in chapter 5.2).

## 6.3 Data processing guidelines

For the in-plot assessment, the percentage of branches in the different frequency classes will be assessed.

For the LESS assessment, the following main data processing is suggested:

Estimates in terms of frequency, means and totals: frequency of quadrates including symptomatic plants (% of forest edge vegetation area affected)

Frequency of symptomatic species (% of symptomatic species over the total number of species of the forest edge)

Mean number of symptomatic species

Total number of symptomatic species

Estimates should be calculated with confidence intervals at a 95% probability level.

The results attributed to LEVEL II sites will be documented in a map covering Europe, characterizing areas of increased ozone risk for European forest ecosystems.

## 6.4 Data reporting

All validated data should be sent to the National Focal Centre and submitted annually to the transnational central data storage. The respective submission forms are provided by the National Focal Centre. An overview of the submission forms is given in Table 8.

**Table 8.** Abbreviation and content of the submission forms for ozone visible injury data

| Form abbreviation | Content description |
|---|---|
| PLL | Plot information for ozone injury assessment |
| LTF | Symptom and sample information for in-plot ozone injury assessment (IM) |
| LSS | Symptom data from off-plot assessment (LESS) |
| OTS | Symptom data from off-plot assessment (LESS-plus) |
| OZP | Photo documentation file names |
| DAR-Q | Contains additional information on local conditions, sampling, transportation and storage procedures, analytical procedures, and interpretation of the results. |

Note: In form LSS, for squares without plants or containing only plants that are not considered in this survey, report the code “Empty” for “Species name”, and the code “000.000.000” for “Species code”.

# 7. References

Directive 2008/50/EC of the European Parliament and of the Council of 21 May 2008 on ambient air quality and cleaner air for Europe. (see http://www.ozoneeffects.org/Downloads_EN)

Flora Europaea code list for vascular plants. (see http://www.ozoneeffects.org/Downloads_EN)

ICP Forests Working Group on Ambient Air Quality. http://www.icp-forests.org/WGaaq.htm.

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# Annex I - Procedure for the establishment of a Level II LESS

For the establishment of the LESS, the following procedure is to be applied:

1. Identify an area (A) (500 m radius) centred around the Level II open-field monitoring station (meteorological tower and/or deposition devices) where passive $O_3$ samplers are installed (M) (Fig. A-1a).
2. Identify all the light exposed forest edges within A (Fig. A-1a).
3. From those, choose the forest edge closest to M (Fig. A-1b).
4. Determine the start point and measure the length of the selected forest edge and virtually identify a 1 m width area along them. You now have an *x* m long and 1 m width transect (Fig. A-1b).
5. Calculate how many possible 2 x 1 m non-overlapping quadrates fit into the selected forest edge area by dividing the *x* m long transect by 2. The 2 m long edge of the rectangular quadrate lies along (parallel) the forest edge. The total number of non-overlapping quadrates is our target population.
6. Select your sampling quadrates randomly.
7. At the end, you will obtain a list of *n* codes. Each code is a 2 x 1 m quadrate within the LESS; the codes will give you the distance of the beginning of each quadrate from the beginning of the previously determined start of the forest edge. Now you are ready for the field to install the LESS (Fig. A-1d).

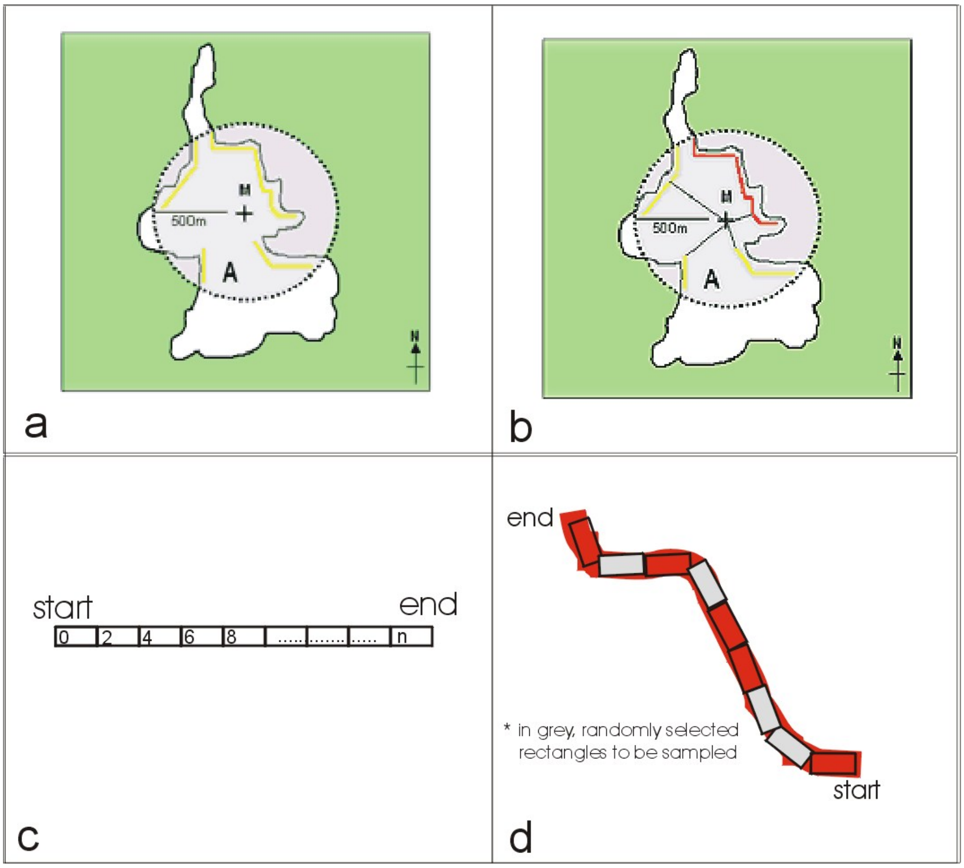



**Figure A-1**. LESS establishment and the selection process of non overlapping quadrates within a light exposed forest edge

*Particular cases*

If there is no forest edge at all, the assessment cannot be conducted. The site must be discarded for the assessment of ozone visible injury.

If there are forest edges only beyond the 500 m radius, the site must be discarded.

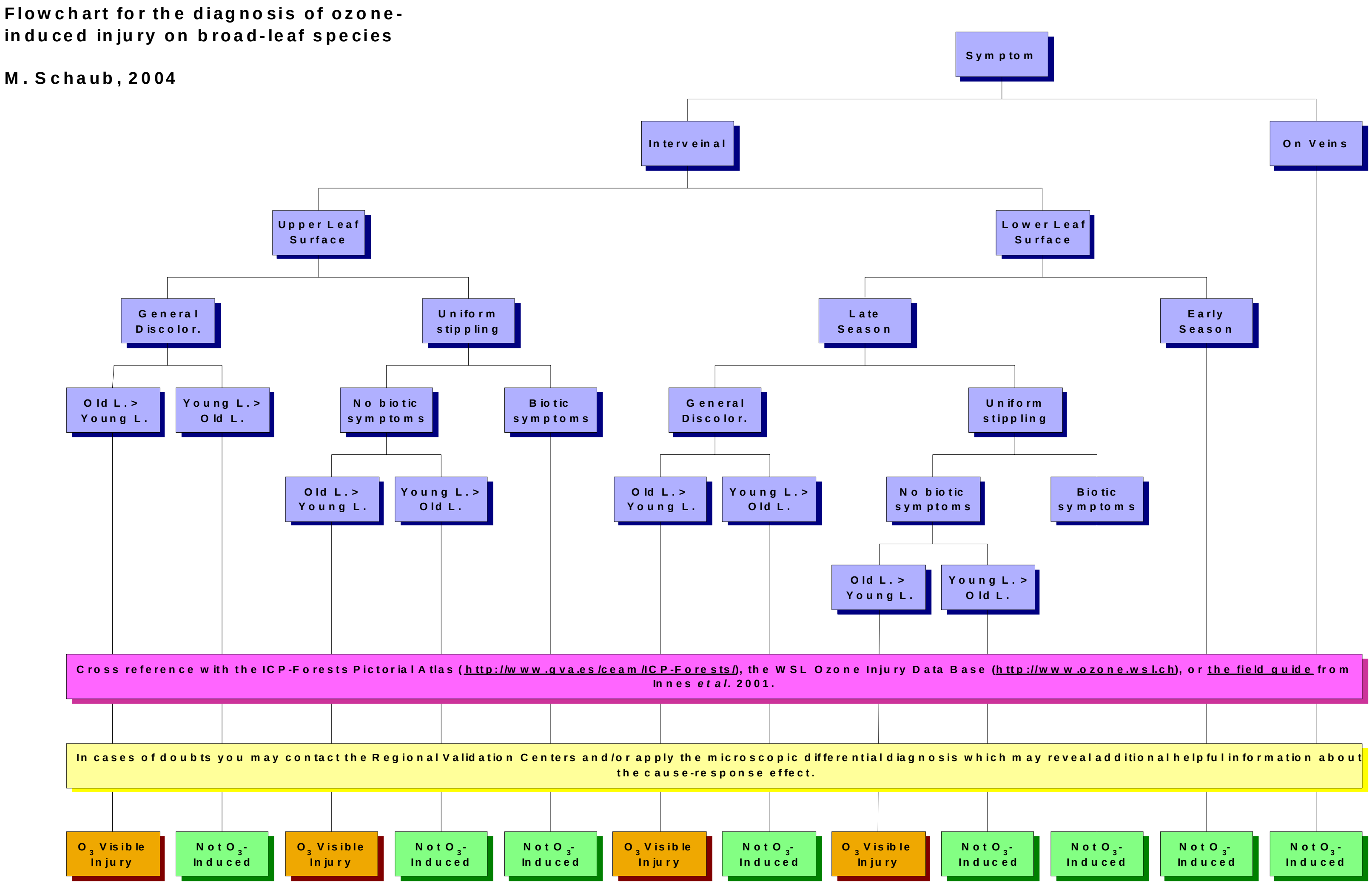
Temporal fashion of LESS

There is no need to permanently mark the LESS if only one annual assessment is planned. For repeated LESS assessments within the same season, the LESS must be marked.

**Table A-1**. Sample sizes at specified precision levels, for different lengths of the selected forest edge.

| Length of light exposed forest edge [m] | Possible 2x1 m non overlapping quadrates [n] | Adjusted sample size (FPC adjusted), 10% error [n] |
|---|---|---|
| 30 | 15 | 13 |
| 35 | 18 | 15 |
| 40 | 20 | 17 |
| 45 | 23 | 18 |
| 50 | 25 | 20 |
| 60 | 30 | 23 |
| 70 | 35 | 26 |
| 80 | 40 | 28 |
| 90 | 45 | 31 |
| 100 | 50 | 33 |
| 150 | 75 | 33 |
| 200 | 100 | 33 |
| 250 | 125 | 33 |
| 300 | 150 | 33 |
| 350 | 175 | 33 |
| 400 | 200 | 33 |
| 450 | 225 | 33 |
| 500 | 250 | 33 |
| 600 | 300 | 33 |
| 700 | 350 | 33 |
| 800 | 400 | 33 |
| 900 | 450 | 33 |
| 1000 | 500 | 33 |
| 2000 | 1000 | 33 |

# Annex II - Flowchart for the diagnosis of ozone symptoms on broad-leaf species



# Annex III - Computer generated chart for the evaluation of chlorotic mottling

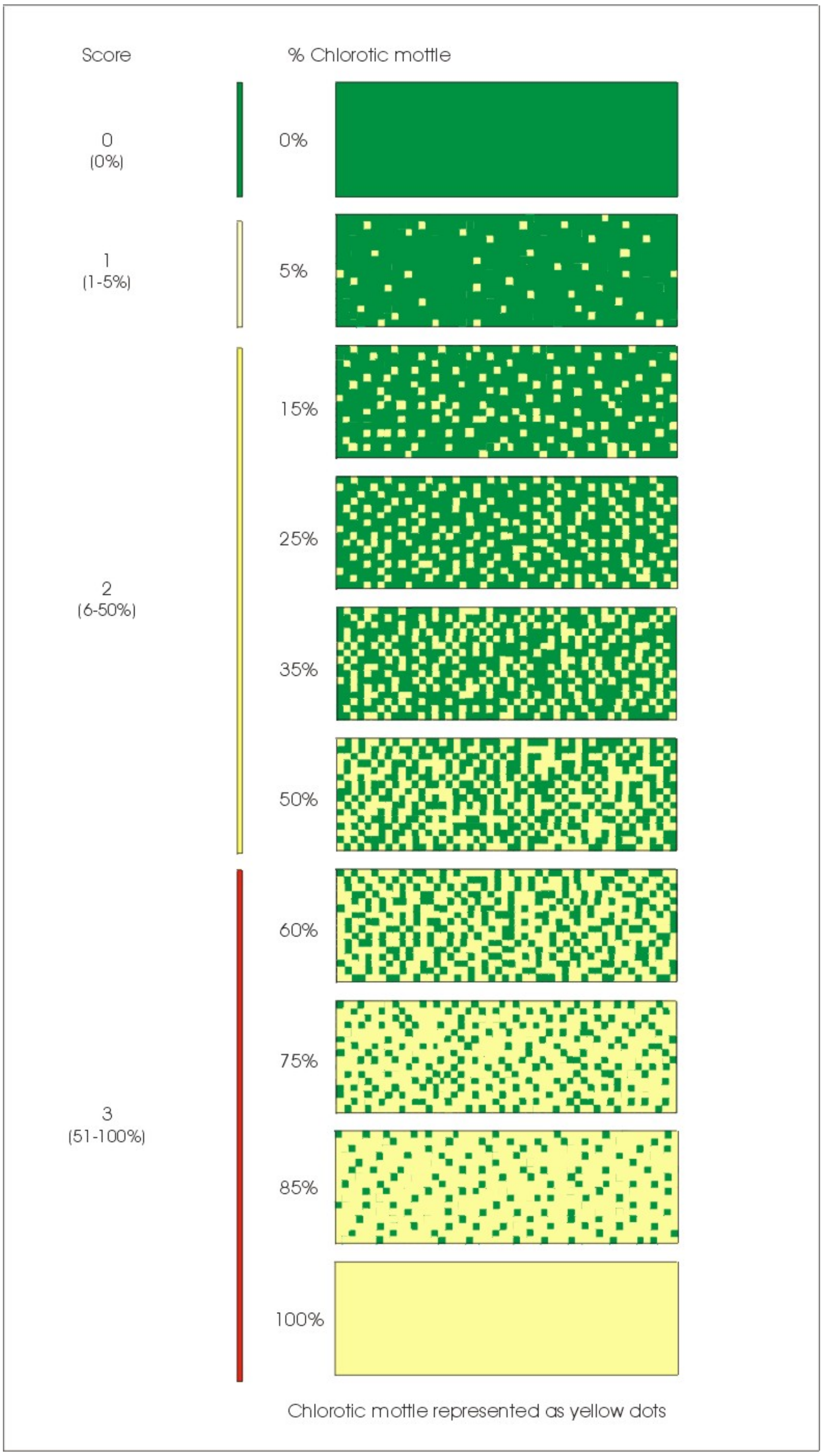



**Figure A-2**. Computer generated chart for the evaluation of chlorotic mottling (V. Calatayud, 2000)

# Annex IV - Guidelines for collecting samples for microscopical analysis

1. Material:
   a) Container: ice box cooler, boxes.
   b) Magnifying lens.
   c) Labels, pencil, scotch tape
2. Tools: a sharpened punching tool (diameter 0.5-1cm), sharp scalpel, cardboard, tweezers, latex gloves, Kleenex.
3. Solutions: fixation medium (2.5% glutaraldehyde in Soerensen buffer at pH 7.0 distributed in 1.5 ml Eppendorf vials with screw caps).
4. Procedures:
5. Excise small samples (max. 0.5-1 cm in diameter for leaves, 3-4 mm for needles) from symptomatic as well as asymptomatic leaves/needles, in order to ease and thus speed up the penetration of fixative,
6. Immediately dip samples into Eppendorf vials with accurate labels (1 disc/needle segment per vial only) prior to storage in the cooler. Unless samples are collected to sort out stress symptoms by different stress factors, multisymptomatic samples should be avoided by close examination of leaves and needles prior to sampling. The hand lens should also be used to select asymptomatic samples from foliage close-by with similar light exposure.
7. Back to the lab, renew the fixative solution and, if possible, remove the air in samples by vacuum evacuation using an excicator connected to a vacuum pump.
8. Store then the samples at 4 °C until processing for microscopical analysis.

Note: Document the symptom morphology and distribution details at leaf/needle level with macro pictures prior to microscopy sampling.

The WG on Ambient Air Quality can be contacted to supervise sampling and microscopical analyses.

# Annex V - Field form for ozone visible injury assessment at Level II LESS

Level II Ozone Injury Assessment_Fieldform

*Plot name* ______

*Date (DD.MM.JJJJ)* ______

*Precision level (%)* ______

*Established quadrates (n)* ______

*LESS length (m)* ______

*Name of surveyor* ______

Symptom codes:

- 0 = no symptoms *
- 1 = ozone symptoms (only) *
- 2 = symptoms due to other (not O3) causes
- 3 = 1 AND 2
- 4 = unknown, not validated *
- 5 = no woody species present

*) For UNECE data base

| *Quadrate ID (# = m)* | | | | | | | | | | | | | | | | | *POSPLAR* | *P. serotina* | *Photo #* | *Comment #* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Quadrate sequence # | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | OTS | | | | |
| *Name of woody species* | Symptom codes | | | | | | | | | | | | | | | | | | | |
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*Comments*

Fieldform_vs4.xls latest update 13.04.10

To be downloaded from [http://www.ozoneeffects.org/Downloads_EN](http://www.ozoneeffects.org/Downloads_EN)