DEvelopment of a quick Monitoring index as a tool to assess Environmental impacts of TRAnsgenic crops (DEMETRA)

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Ente Parco Regionale Migliarino San Rossore Massaciuccoli

UNIVERSITÀ DEGLI STUDI FIRENZE
GESAAF
DIPARTIMENTO DI GESTIONE DEI SISTEMI AGRARI, ALIMENTARI E FORESTALI

DEvelopment of a quick Monitoring index as a tool to assess Environmental impacts of TRAnsgenic crops (DEMETRA)

Preface

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In 2008 the Community program LIFE + gave the opportunity to present projects to develop and deepen the theme of the environmental risk assessment of Genetically Modified Organisms (GMOs), through the development of specific environmental monitoring systems. At that time Italy was outlining a legislative framework which suggested the need to define the rules of coexistence to fulfill its obligations under Community law and comply with the national legislation in force (Law n.5/2005). In this regulatory framework, the Tuscany Region promoted the idea of developing a LIFE + project on the theme of GMOs monitoring, to try to fill the gap related to the lack of standardized tools to carry out environmental monitoring of GMOs and, at the same time, respond to the need to protect its territory from risks determined by transgenic crops.
The DEMETRA (DEvelopment of a quick Monitoring index as a tool to assess Environmental impacts of TRAnsgenic crops) project contributes to building a shared basis at Community level for the monitoring of GMOs into the environment, whether they are directly cultivated for commercial purposes, whether they are used in a given place for research purposes.
The main objective of the Project was the creation of an innovative instrument to rapidly address monitoring efforts that Public Bodies should implement in their territories when transgenic crops are cropped. Particularly on when, where and how the data collection should be switched on.

Where, when and how to address the efforts of Public Authorities in the General Surveillance for the monitoring of possible collateral effects of Genetically Modified Plants (GMPs) is the main objective of this project.
An objective that is particularly relevant for those public bodies who have to manage directly those issues related with the commercial cropping of GMPs (as in the case of Italian Regions).
This project has been developed starting from the assumptions highlighted in the "Outcomes of the EC Working Group on Guidance Notes supplementing Annex VII of Directive 2001/18/EC" (F. Graef, A. De Schrijver, B. Murray), which indicate that:

- there is no guidance as to how existing monitoring programs and data infrastructure

schemes may support GMO monitoring,
- there is no legal framework to regulate the coordination and harmonization of GMO monitoring data,
- monitoring data should include standardized numerical raw data ready to be analyzed with an informative system.

The DEMETRA project aims particularly at addressing the latter bullet point, with the creation of a quick monitoring index (QMI) to rapidly assess the potential risk generated by a selected range of transgenic crops in well determined ecosystems or biotopes.
The index will take into account:
- the level of risk posed by a range of transgenic crops potentially used in the study areas and
- the potential interactions of these GMPs with some relevant biological, physical and climatic parameters that will be collected and studied in some sites of the study areas.

The index has been equipped with a Geographic Information System (GIS) which, provided with geographic data, will be useful to monitor and map the level of risk generated by transgenic plants in a determined area, either these GMPs are really cropped or that their presence is only simulated.

Project outputs

The QMI and GIS platform provided with necessary data, can be used as a tool to map the level of environmental risk posed by transgenic crops to specific ecosystems.
Moreover, the project enlists guidelines to correctly choose the elements to be considered during the setting up of a monitoring system.
In addition, this project has given the possibility to generate a starting point for biodiversity in areas where transgenic crops have never been used. The resulting data-sets, reported in the chapters of this book, can be used when transgenic crops enter in common use, allowing a comparison among observed parameters linked to biodiversity.
The system has been studied to be really transferable in other situation, but it will work effectively only where a wide range of environmental information is already collected, stored and easy available for Environmental Risk Assessment (ERA) and GIS applications.
The project has contributed also to the objectives of the Commission Communication COM (2006) 216 final: ”Halting the loss of Biodiversity by 2010 – and beyond” with particular regard to:
- Policy area 1:

Objective 1 “To safeguard the EU’s most important habitats and species”, as the project was aimed at define correct GMO monitoring system for sensitive areas;
Objective 5 “To substantially reduce the impact on EU biodiversity of invasive alien species and alien genotypes”, as the project was aimed at individuate particular risks in the use of GMOs, which can be considered as “alien genome”;

- Policy area 4:

Objective 10 “To substantially strengthen the knowledge base for conservation and sustainable use of biodiversity, in the EU and globally”, as the project was aimed at directly improve the knowledge on biodiversity and biodiversity trends.

To reach its objectives the project has foreseen the following activities:

- Collecting, analyzing and selecting already known parameters linked to weather local conditions, soil functionality, trophic chains, landscape uses, biodiversity and biodiversity loss to generate a model, which is the basis for the generation of a Risk Assessment method.
- Assessing the suitability of data collected with the most relevant monitoring systems and selecting the most relevant ones for the definition of the quick monitoring index (QMI).
- Developing the index so to express the potential perturbation that transgenic crops could pose to a certain ecosystem or biotope in condition of different intensity of cultivation.
- Identifying and creating specific study sites to generate simulations of the application of the QMI.
- Creating a GIS platform to run the modeling system.
- Developing guidelines and best practices to apply monitoring schemes in high risk areas.

Furthermore, the project is expected to contribute and to improve the knowledge for the development of European policies to prevent risks in the commercial use of GM crops.

Chapter 1

Description of the Studied Areas

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The studied areas are located in the Regional Park of Migliarino – San Rossore – Massaciuccoli, in the Pisa province (Tuscany, Italy) (Fig. 1). The protected area is characterized by a wide diversity of ecosystems and a high presence of lands suitable for agriculture. The project focused on three study areas characterized by different ecosystems.

Area 1 (Massaciuccoli Lake)

Fig. 1. Map of the project study area.
1. Wetland area of Massaciuccoli Lake;
2. Natural poplar forests and non cultivated areas;
3. Mixed and pine forests, wetlands, cropped area.

The "Lago e Padule di Massaciuccoli" (Cod. Natura2000 IT5120021) covers a total area of 1,908.01 ha, and it is a "Site of Community Importance" (SCI) according to the directive 92/43/CEE. Additionally, the SCI overlaps the "Site of Regional Importance" (SIR) according to the Regional Law L.R. 56/2000.

This site is entirely included in the Regional Park of Migliarino – San Rossore – Massaciuccoli and in the contiguous area. A protected area managed by LIPU (Italian League for Protection of Birds) is inside the site here considered.

The land of the project area is designed to agriculture and it falls in a private property.

Lake of fresh water surrounded by helophytic formations (reeds and *Cladium* spp.), bog and upland vegetation. The area is also characterized by upland forests, deep puddles of water belonging to sandy extraction. It is characterized by phytocenosis with *Cladium mariscus* and by other rare species, such as *Periploca graeca*

(one of the few Italian stations), and *Drosera rotundifolia* a very rare upland species in the bog.
Massaciuccoli Lake and its surrounding marsh area is one of the most important wetland habitats in Italy. It is characterized by a large lake slightly deep, on average less than two meters, with obvious problems of eutrophization and wide marsh areas, particularly in northern part of the site.
The habitats of community interest (Annex I Dir. 92/43) are small depressions on peat substrate with a community of Rhynchospora alba and/or *R. fusca* (code 71.50) and the neutral-basophils peat bogs with a dominance of *Cladium mariscus* and/or *Carex davalliana* (code 72.10). Among the flora species *Marsilea quadrifolia* is included in the Annex II Dir 92/43.
The animal species are much more numerous; it is a very important site for migratory and wintering species of birds. Among the breeding species of greatest importance is certainly the bittern (*Botaurus stellaris*), for which the lake is the most important nesting area at national level; then, we underline the presence of *Ixobrychus minutus, Ardea purpurea, Himantopus himantopus, Circus aeroginosus and Acrocephalus melanopogon*. All these species are related mainly to the marsh areas and in particular reeds.
The amphibian *Triturus carnifex*, an Italian endemism, and the reptile *Emys orbicularis* are species of Community interest, cited in Annex II of Dir. 92/43/CEE.

Area 2 (Serchio River) and Area 3 (Arno River)

These study areas are in the SCI/SIR/SPA “Selva Pisana” (Cod. Natura2000 IT5170002) with a total area of 9,658.34 ha. This site is entirely included in the Regional Park of Migliarino – San Rossore – Massaciuccoli.
The land of the project area falls in the public property of the Tuscany Region and it is managed by the Regional Park of Migliarino – San Rossore – Massaciuccoli.
Sandy coast, mostly in regression, is characterized by the typical sequence of plant formations of sand heaths (*Cakile maritima* community, seseleto, *Elymus farctus* community, *Ammophyila littoralis* community, *Phragmites australis* community and gen. *Juniperus* community). The area is composed by both coastal dune habitats and internal system of fossil dunes and interdunes with alternance of pine forests with *Pinus pinaster* or *Pinus pinea*, high bush, freshwater and brackish wetlands, wide forests with *Quercus robur*. The internal wetlands are characterized by mosaic of *Salicornia* sp.pl. community, plant formations of helophytes, such as *Phragmites australis* community e *Carex* sp.pl. community, and former farmland flooded in winter.
Dunal ecosystems and humid retrodunal areas host habitats and species of flora and fauna with high conservatory interest.
One of the 2-3 best examples of dunal habitat in Tuscany, slightly influenced by man, is included in the considered area. Dunal habitat of this area represents one of the best ones along the Tyrrhenian coast and, together with the neighbouring site “Coastal Dune of Torre

del Lago", the only one in a good state of conservation in the northern Tuscany.
These considerations are related to habitats of UE interest, such as "Embryonic shifting dunes", "Shifting dunes along the shoreline with *Ammophila arenaria* (white dune)", "Annual vegetation of the lines of marine deposit", and to priority habitats, such as "Coastal Dunes with *Juniperus* spp." and, in back-dune area, "*Calcareous fens* with *Cladium mariscus* and *Caricion davallianae* spp.".
The wetlands, included in the area here considered, are interesting at National and sometimes at International level, for the wintering of waterfowl and for the rest during migration along the Tyrrhenian coast route.
Dunal and retrodunal habitat host rare plant species, such as *Solidago virgaurea* spp. *litoralis* (an endemism of versiliese-pisano sandy coast), *Stachys recta* var. *psammofila* (endemism of the Tyrrhenian coast), *Periploca graeca* (one of the few Italian stations), and several nesting populations of UE interest species (in particular *Burhinus oedicnemus*). Reproductive populations of *Rhinolophus ferrumequinum* and *Myotis emarginatus*, species included in the Annex II of Dir. 92/43/CEE, live in the considered area. *Rhinolophus ferrumequinum*'s colony is the only one known in Tuscany and the biggest in Italy; some individuals of this colony winter in a building inside the site.

In each area, permanent study areas and, within them, the sub-areas (transect, plot, etc.) needed for data collection have been identified (Table 1).

Table 1. The study areas.

	Locality	System – sub-area	Sampling sites
AREA 1 (Massaciuccoli Lake)		Lake	6 points for biodiversity 32 poplar
		Poplar plantation	30 poplar
AREA 2 (Serchio river)	Ontanelli	Not cropped area-parcel 821	21 sub-plots
	Fortino nuovo	Natural poplar forests	21 sub-plots 30 poplar
	Fortino nuovo	Poplar plantations	30 poplar
AREA 3 (Arno river)	Culatta	Cropped area-Parcel A6	Crops
	Culatta	Mixed forest	43 sub plots, 267 *loro campestre*
	Colmate del Bozzone	Pine forest	7 sub-plots
	Colmate del Bozzone-lame	Wetland area	10 plots

Vegetational description of the sampling site of the permanent study areas

During the first months of operation plot/sampling points, some plants and trees (poplar and maple) of interest for the project have been defined. The plots were staked, the plants marked with labels, and the geographic coordinates of the plots were determined using a GPS and registered in a GIS. The plots of study were defined according to the scheme of Dengler (2009) using a variable number of modules based on the extension of the area under examination. In particular, each module has a squared area of 1,000 m^2 with smaller areas of 100 m^2 and 10 m^2 along the main diagonal of the square. Within these

squares investigations of plant biodiversity, animal and microbial have been carried out (Chapter 4). The identification of permanent plots allowed to repeat the investigations of biodiversity in the same points for the duration of the project allowing to compare data between different years.

Study Area 1 (Massaciuccoli Lake)

Massaciuccoli Lake has an extension of 2,000 hectares and is the most important wetland area in Tuscany. The particular climate of this area has allowed the survival of relict vegetation. The bird population is very diversified. The lake is an important site for nesting and resting place for migratory species: in fact there are over 260 birds species. The study area, called "Anghetto", is approximately 30 ha and is located near Massaciuccoli in the municipality of Massarosa. It is bounded NW from Massaciuccoli Lake and the rest by canals (ditch Navicello) communicating with the lake itself. The vegetation is mainly made up of *Phragmites australis* (reed) in parts of the border, with the lake and the canals, and *Cladium mariscus* (sedge) in the central parts.

Fig. 2. Study Area 1 (Massaciuccoli Lake).

The whole area is almost always flooded except the outer edges, embankments, and some points raised where some examples of spontaneous poplars are also present.

In addition to the straw and the sedge, the vegetation of this environment is characterized by the presence of *Solanum dulcamara, Iris pseudoacorus, Osmunda regalis, Hibiscus palustris and Periploca graeca*.

In the waters of the canals and of the lake, *Ceratophyllum demersum, Potamogeton pectinatus, Utricularia australis, Lemna gibba and Myriophyllum spicatum* are common species.

Because of the particular type of the site, "points" for sampling, distributed on the area south of the lake, have been identified and in which the investigation of plant (grasses) and animals biodiversity were made. In addition, the poplar individuals were identified to study the genetic diversity and the gene flow (breeding) with a poplar plantation present near the southwest edge of the lake (Fig. 2).

Study Area 2 (Serchio river)

- Natural poplar forest (Fortino nuovo Locality)

The area includes a mixed forest characterized by the presence of White poplar (*Populus alba* L.), Elm (*Ulmus minor* L.), Narrow-leafed Ash (*Fraxinus oxycarpa* Bieb.), Black alder (*Alnus glutinosa* L.), Grey poplar (*Populus x canescens* ((Aiton) Sm.)) and some individuals of peduncolate oak (*Quercus robur* L.). This training is similar to an irregular high forest

in which both individuals from seed or individuals developed from the stump are present. The coverage is uneven and regeneration is not present.
This area has a flat morphology, without rockiness outcropping. It is also easily accessible. It is approximately 7,400 m^2 large in which 3 plots have been identified. Moreover, a total of 30 poplar have been identified and the sex phenologically determined. Among these 6 female trees have been identified (Fig. 3).
An area of 2,500 m^2 was delimited in order to determine the qualitative and quantitative characteristics of the forest stand.
- Not cropped area - parcel S21 (Ontanelli Locality)
This is an area in which cultivation is not carried out since 10 years. This sub-area has a surface of approximately 14,000 m^2 in which 3 plots have been identified (Fig. 4).

Fig. 3. Study Area 2 (Serchio river): natural poplar forest (Fortino nuovo Locality).

Fig. 4. Study Area 2 (Serchio river): not cropped area – Parcel S21 (Ontanelli Locality).

Study Area 3 (Arno river)

- Mixed broadleaved forest (Culatta Locality)

The study area is located on a flat terrain, without rockiness outcropping. Furthermore, being situated on the edge of a forest road it is easily accessible.
The study area borders a mixed broadleaved forest characterized by the presence of Peduncolate oak (*Quercus robur* L.), Narrow-leafed Ash (*Fraxinus oxycarpa* Bieb.), Gray poplar (*Populus* x *canescens* ((Aiton) Sm.)), maple (*Acer campestre* L.), Common hornbeam (*Carpinus betulus* L.), elm (*Ulmus minor* L.), Black alder (*Alnus glutinosa* L.) and White poplar (*Populus alba* L.).
Forest cover is multi strata with oak trees of considerable size and younger individuals of other species with widely varying dimensions. The stand density is not uniform. Crown cover is uneven with openings due to uprooted plants. The undergrowth is characterized by the significant presence of elmleaf blackberry (*Rubus ulmifolius* Schott) and common hawthorn (*Crataegus monogyna* Jacq.).
The regeneration is limited probably due to the excessive load fauna that persists over the area. This formation lies in the association of *Fraxino angustifoliae-Quercetum roboris* Gellini, Pedrotti, Venanzoni 1986.
In this sub-area of about 118,000 m^2, 6 plots were selected and two further plots of only 100 m^2 were identified near the edge of the forest. In addition, a total of 267 individuals

of *Acer campestre* (open pollinated mainly entomophilous) were detected. Of these, 32 were then chosen so as to cover up significantly the distribution area to conduct genetic variability study (Fig. 5). An area of 2,500 m^2 was carried out in order to determine the qualitative and quantitative characteristics of the forest area (Fig. 5).

Fig. 5. Study Area 3 (Arno river): mixed broadleaved forest (Culatta Locality).

- Pine forest and wetland area (Colmate del Bozzone Locality – Lame)

In this area two types of vegetation formation were considered.

The first type is a pure Italian stone pine forest (*Pinus pinea*) 100-120 years old. Pine trees have large crown which lead to an almost complete ground cover. The undergrowth is mainly composed by the herbaceous layer of grasses.

The second type of vegetation is an open area characterized by a large grassy area that most likely comes from reclaimed marshlands.

These two previous situations are divided by a thick bed of reeds.

In this sub-area of about 25,000 m^2, one plot has been identified in the pine forest and an area of 2,500 m^2 was carried out in order to determine the qualitative and quantitative characteristics of the forest stand.

Fig. 6. Study Area 3 (Arno river): Pine forest and wetland area (Colmate del Bozzone Locality – Lame).

Additionally 10 plots each of 100 m^2 have been identified in the wetland area adjacent to the pine forest (Fig. 6).

- Cropped area - parcel A6 (Culatta Locality):

This area is located within an area used for agricultural from MSRM Regional Park, where it was not possible to identify the permanent study plots as not to hinder the activities of the Park. In this area, maize, sunflower and oil-seed rape were cropped for the study (Fig. 7). In particular, the investigations pollen flow and breeding between the oil seed-rape cultivated and wild relative were conducted.

Fig. 7. Study Area 3 (Arno river): cropped area - parcel A6 (Culatta Locality) in which maize, sunflower and oil-seed rape were cropped. cultivation in 2012 year.

Characterization of forest structure

In order to characterize the species composition and the structure of forest formations in the Park of MSRM, forest structure was investigated in the following study areas: “Natural poplar forest” (Area 2), “Mixed broadleaved forest” (Area 3), “Pine forest and wetland area” (Area 3). In each sub area, field works were carried out within a squared plot 2500 m^2 large, and the positioning of living trees, dead trees (standing dead trees and lying dead trees), and dead wood on the ground was determined.

For living trees, the species was noted and the following parameters were measured: the diameter at breast height (DBH), the total tree height, the height of base crown, and the projection of the crown in 4 directions (N, E, S, O). For dead trees the species was noted and the following parameters were measured: the DBH, the total tree height, and the decay class. For pieces of dead wood on the ground and for stumps, the species was noted and the following parameters were measured: diameter at the two ends, the total length (the height in case of stumps), and the decay class.

The characterization of flora and fauna in the forest ecosystems was supplemented with an analysis of the specific composition and spatial structure of forest stands in order to have a set of useful data for the development of indicators for monitoring and for their implementation map-based systems with GIS.

Table 2 shows the dendrometric parameters for “Natural poplar forest” (Study Area 2 Serchio river, Fortino nuovo Locality). The stem number-diameter distribution in diameter classes of 5 cm is shown in Figure 8.

Table 2. Natural poplar forest: number of trees, basal area and volume per hectare.

Species	Number n/ha	Basal area m^2/ha	Volume[1] m^3/ha
Maple	4	0.1	0.4
Narrow-leafed Ash	44	8.0	138.3
Elm	28	1.4	10.1
Black alder	188	8.0	77.7
Poplar	20	8.0	141.7
Total	**284**	**25.4**	**368.2**

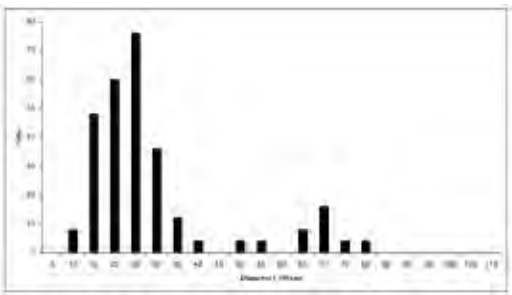

Fig. 8. Stem number-diameter distribution for “Natural poplar forest”.

Table 3 shows the dendrometric parameters for “Mixed broadleaved forest” (Study Area 3 Arno river). The stem number-diameter distribution in diameter classes of 5 cm is shown in Figure 9.

Table 3. *Mixed forest: number of plants, basimetric area and volume per hectare.*

Species	Numer n/ha	Basal area m^2/ha	Volume^2 m^3/ha
Maple	44	1.3	11.3

Species	Numer n/ha	Basal area m^2/ha	Volume^2 m^3/ha
Whitethorn	20	0.3	0.6
Peduncolate oak	28	9.5	151.5
Narrow-leafed Ash	436	15.4	192.5
Elm	80	1.6	11.7
Total	**608**	**27.9**	**367.5**

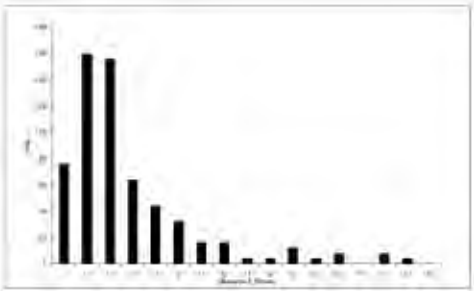

Fig. 9. Stem number-diameter distribution for “Mixed broadleaved forest”.

Table 4 shows the dendrometric parameters for Italian stone pine forest. The distribution of the number of trees in diameter classes of 5 cm is shown in Figure 10.

Table 4. *Pine forest: number of trees, basimetric area and volume per hectare.*

Species	Number n/ha	Basal area m^2/ha	Volume^2 m^3/ha
Pine	92	33.2	517.9
Total	92	33.2	517.9

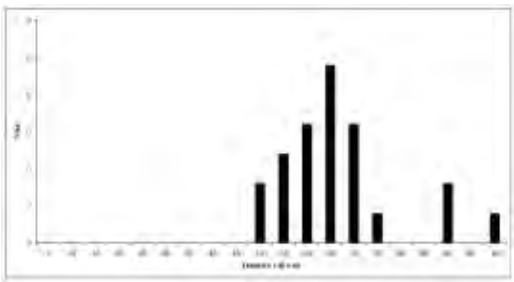

Fig. 10. Stem number-diameter distribution.

Crops done from 2010 to 2012

The crops of maize, sunflower and oilseed rape made and used during the project are summarized in the following table:

Locality	Crops in 2010	Crops in 2011	Crops in 2012
1. Massaciuccoli Lake			Private poplar plantation
2. Ontanelli, plot S23		Poplar plantation presents in MSRM	
3. Fortino Nuovo		Poplar plantation presents in MSRM	

Locality	Crops in 2010	Crops in 2011	Crops in 2012
4. Culatta, plot A6	Maize; Sunflower	Maize; Sunflower; Oil seed-rape*	Maize; Sunflower;
5. Culatta, plot A9		Maize	
6. Migliarino (close to A11)	Oil seed-rape* (cropped by the private Marchese Mazzorosa)		

* The oil seed-rape is autumnal cultivar, therefore it is seeded in August/September and the pollen production occurs in the spring (March/April) the following year.

Types of weather stations

In consideration of the needs of the project, the weather stations have different characteristics according to the study area in which they are located (Fig. 11):

1. Weather station 1 (Area 1 - Massaciuccoli Lake) measures the following parameters:
- Intensity and wind direction at a height of 4 m above the lake;
- Temperature and humidity of the air at a height of 3.5 m above the lake;
- Rain.

2. Weather Station 2 (Area 2, Ontanelli locality) measures the following parameters:
- Intensity and wind direction in three installments (2.5 m, 5 m, and 10 m);
- Air temperature and humidity at 2 m;
- Temperature and soil moisture at a depth of 10 cm;
- Rain.

3. Weather Station 3 (Area 3, Culatta locality) measures the following parameters:
- Intensity and wind direction in three installments (2.5 m, 5 m, and 10 m);
- Air temperature and humidity at 2 m;
- Temperature and soil moisture at a depth of 10 cm;
- Rain.
- Atmospheric pressure
- Solar radiation (global, diffuse, sunshine, UVA, UVB)

[1] The volume was computed using double entry volume equations (I.F.N.I., 1985).
[2] The volume was computed using double entry volume equations (I.F.N.I., 1985)
[3] The volume was computed using double entry volume equations (I.F.N.I., 1985)

Every 15 minutes the average, or the sum for rain and sunshine duration is performed and stored.
The power supply of tools and acquisition systems is provided by photovoltaic panels with buffer batteries.

Fig. 11. Images of the weather stations in the 3 study areas.

Chapter 2

Pollen flow and breeding evaluation

2.1 - Pollen Flow

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Introduction

Information on pollen flow are necessary to assess the potential impact on ecosystems of genetically modified organisms (GMOs). For instance, pollen dispersal data are needed to evaluate the range within transgenic pollen could affect biodiversity and non-target species. This knowledge is also necessary to identify environmental conditions that might favour pollen movement. Additionally, information on pollen flow can be used to estimate the range of the pollen dispersal using dispersal simulation models (see chapter 5).

Several studies have analyzed maize pollen flow and deposition using pollen traps (e.g., Brunet et al., 2003; Devos et al., 2005; Walklate et al., 2004) while other have investigated pollen flow distance for poplar using molecular markers (e.g., Burczyk et al., 2004; DiFazio et al. 2004).

Several pollen dispersal simulation models have been proposed in the last years (e.g., Lavigne et al., 1996; Colbach et al., 2001a, 2001b; Klein et al., 2003; Balducci et al., 2007; Mazzoncini et al., 2007). However, these models do not consider relevant features like elevation and the presence of natural and artificial barriers (e.g., hedges, windbreaks) that can play an important role in pollen filtration/dilution.

Our work focuses on pollen dispersal data of the following species (crops and trees) of which genetically modified variants can be commercially available in the near future: maize (*Zea mays* L.), oilseed rape (*Brassica napus* L.), sunflower (*Helianthus annuus* L.), and poplar (*Populus nigra x Populus deltoides*). We used pollen traps to assess the distances covered by pollen granules of the selected species and a pollen dispersal simulation model to assess the potential contamination levels due to maize crops. At least to our knowledge,

this is the first study that attempt to obtain data on pollen dispersal for sunflower, oilseed rape (wind and insect pollinated plant) and forest trees such as pine and poplar (wind pollinated trees) using pollen samplers. It is worth noting that even in the case of insect pollinated plants, wind pollination - though often negligible - is a component which should also be analyzed.
Moreover, considering the importance of insects in the transport of pollen, monitoring of the pollen flow by bees (*Apis mellifera*), insect pollinator, was carried out during the entire period of sunflower blooming in the experimental campaign of 2012.

Material and methods

Pollen dispersal data were collected in 2011 and in 2012. The experimental plots were selected in the Migliarino – San Rossore – Massaciuccoli Regional Park (Tuscany, Italy) and were characterized by different cropped areas: maize, sunflower, oilseed rape, and poplar.
A meteorological station was installed in the study area. Air temperature and air humidity were measured at a height of 2 m, while wind speed and wind direction were measured at the height of 2.5 m by means of ultrasonic anemometers. All parameters were recorded every minute and averages were calculated and memorized every 15 minutes.
The study on pollen dispersal was carried out to assess the maximum distance which the pollen can reach in the study area. To do this, the traps were installed from the border of the cropped areas at increasing distance taking into account the main wind direction during pollenation (Figs. 1 and 2). The position of the cropped areas and the position of the traps were recorded using a GPS (Global positioning System) receiver.
The Sigma-2 pollen samplers (Verein Deutscher Ingenieure 2007) were used as they are appropriate for environmental monitoring of GMO. The Sigma-2 sampler provides a standardized sampling method for direct microscopic pollen analysis, as the pollen adhering to the deposition area is directly analyzed with regard to species and amount by means of light microscopy. An example of the preparation of pollen trap substrates with strips “Silkostrip (Lanzoni srl)” for pollen collection is shown in Fig. 3.

Fig. 1. Positioning of pollen traps for maize and sunflower in Culatta locality in 2011 (a); for oilseed rape in Migliarino locality in 2011 (b); for poplar in Ontanelli locality in 2011 (c). The red dots represent the pollen traps.

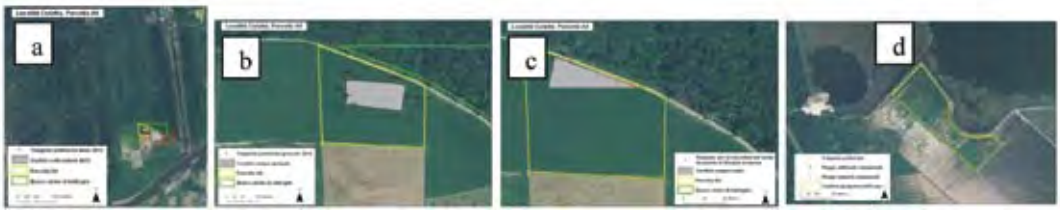

Fig. 2. Positioning of pollen trap for maize (a), for sunflower (b), for oilseed rape (c) in Culatta locality in 2012; and for poplar in Migliarino Lake in 2012. The red dots represent the pollen traps in (a), (b) and (c); the yellow dots represent the pollen traps in (d).

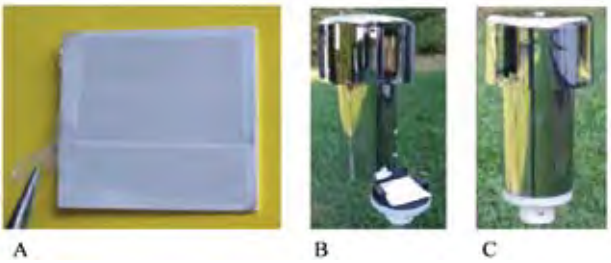

Fig. 3. A: preparation of substrates with strips "Silkostrip (Lanzoni srl)" for the pollen collection; B: the strips rest on the bottom of the pollen trap; C: the Sigma-2 pollen samplers ready for use.

Pollen species were differentiated on the basis of their morphological and structural characteristics distinguishing features such as size, shape, texture, number of pores, structure, etc.

Photos were taken from each plate. On each plate, three strips were placed. After removal from the trap, the strips were stuck on a microscope slide divided into eight rectangles of 1 cm^2 (5 cm x 0.2 cm). The pollen granules count was carried out on each rectangle. A total of 32 readings per sampling point were carried out. Pollen concentration was computed on the basis of the number of pollen grains visualized on the strips (n. granules/cm^2). For each sampling site the pollen counting data were processed and the concentration estimated as readings average (+/- standard deviation).

To study the pollen flow by insect pollinator *Apis mellifera*, eight bee hives were installed at different distances (1 km, 2 km, 3 km) from the experimental field of sunflower, and each hive represented a sampling station of the pollen (Fig. 4). In this way, it was possible to estimate the distance at which the pollen can be transported by pollinating insects.

A specific pollen trap was installed in each hive allowing to sample the pollen collected

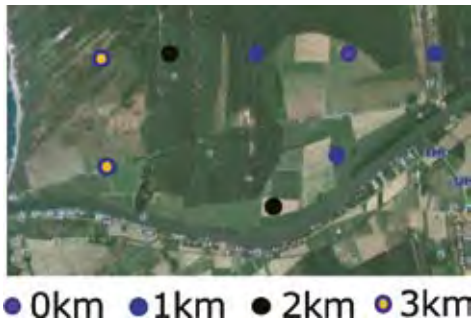

Fig. 4. Positioning of hives at increasing distances from the sunflower cultivation located in Culatta.

Fig. 5. Positioning of the hive near the sunflower cultivation located in Culatta, and pollen sampling.

by bees (Fig. 5). The sampling of the pollen was made every two days for a total of five repetitions, for a total of 40 samples.
The presence of pollen grains in the pollen trap from the cultivated sunflower was assessed by molecular analyses using nuclear microsatellites (nSSRs).

Results

In 2011, maize pollen granules were found up to a distance of 160 m (12 pollen granules/ cm^2 in the farthest pollen trap); the highest number of pollen granules occurred at 0 m, decreasing steeply up to 20 m(87 to 15 pollen granules/cm^2). From 20 m up to 160 m the number of pollen granules remained nearly constant (Fig. 6a). The trend of pollen concentration in relation to distances (in meter) was similar in 2012 (Fig 6a), maize pollen concentration tends to zero at different distances, and farther than 300 m it was not found (Fig. 6 b-g).

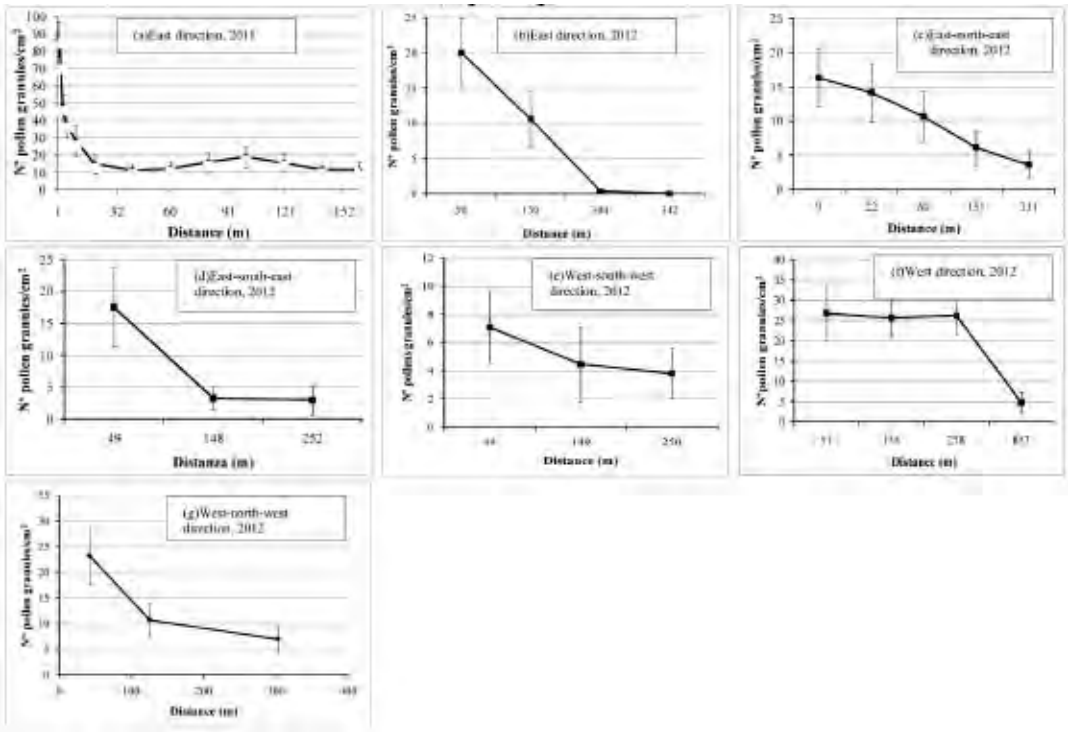

Fig. 6. Pollen flow trend in relation to the distance from cropped areas for maize according to wind direction recorded in 2011 (a), and in 2012 (b-g). Each point represents the means ± standard deviation of the means.

Oilseed rape is an insect as well as a wind pollinated plant. A relevant number of pollen granules of oilseed rape were detected up to a distance of 34 m (49 pollen granules/cm^2 in the farthest pollen trap). The rate of the number of pollen granules steeply decreased

between 0 m and 5 m (271 to 121 pollen granules/cm^2). For oilseed rape, the number of pollen granules remains nearly constant up to 18 m and farther the value is halved to 49 pollen granules/cm^2 (Fig. 7 a-b). A similar trend was detected in the cultivated area of "Culatta" in 2012 (Fig, 7 c-d).

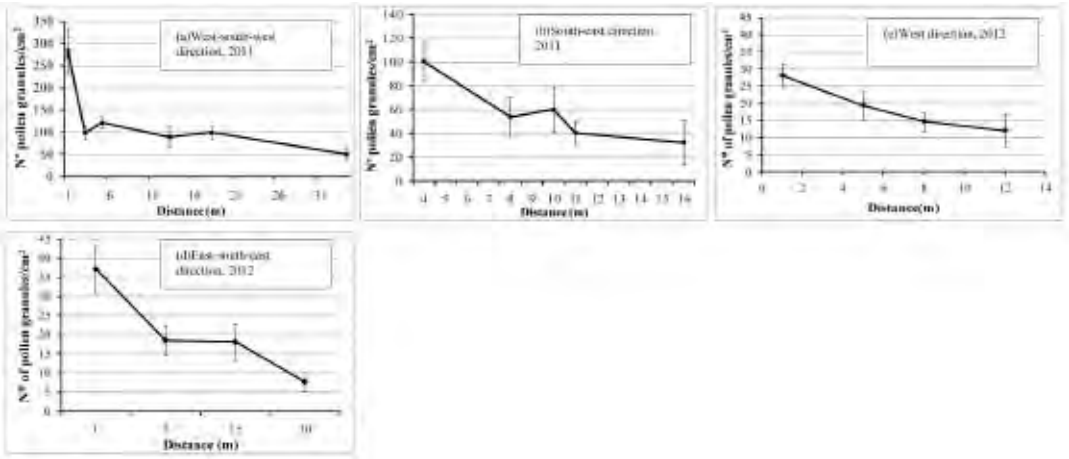

Fig. 7. Pollen flow trend in relation to the distance from cropped areas for oilseed rape according to wind direction recorded in 2011 (a-b), and in 2012 (c-d). Each point represents the means ± standard deviation of the means.

Even if sunflower is an insect pollinated plant, we found wind drifted pollen granules up to a distance of 19 m (23 pollen granules/cm^2 in the farthest pollen trap), with the highest decreasing rate of the number of pollen granules occurring from 0 m to 10 m (from 161 to 18 pollen granules/cm^2). In case of sunflower, the number of pollen granules remained nearly constant up to 19 m (Figure 8c). These results have been confirmed in 2012.

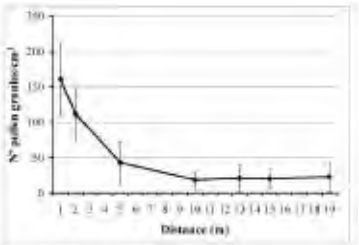

Fig. 8. Pollen flow trend in relation to the distance from cropped areas for sunflower in north-east direction recorded in 2011. Each point represents the means ± standard deviation of the means.

For poplar we found a considerable presence of pollen up to a distance of 380 m in 2011 and up to a distance of 540 m in 2012, which were the farthest pollen traps positioned. The number of pollen granules remains nearly constant (about 350 pollen granules/cm^2) independently from the distance.

Conclusions

The results achieved with the pollen samplers are essential to collect data concerning the distance covered by hypothetical transgenic pollen that could impact on biodiversity and non-target species.
Several studies have been carried out on pollen dispersal of maize considering the measurements of pollen concentrations at various distances and heights from a pollen source. Overall, these studies show that most of the pollen occur within 30–50 m from the source. However, when convective air currents have been considered, the presence of pollen has been observed up to 650 m from a known GM source (Devos et al 2005).
The distance over which the pollen can be dispersed depends on the local environmental conditions as well as on the prevailing climatic conditions (wind direction, humidity, temperature, etc.) The range of pollen dispersal has therefore to be evaluated using a case by case approach. Our results show that the pollen of maize was detected up to a distance of 300 meters from the cultivated area.
Molecular marker have been used for gene flow investigations of oilseed rape, sunflower, and poplar (e.g., DiFazio et al., 2004; Damgaard and Kjellsson, 2005; De-Lucas et al., 2008, Ureta et al., 2008). For the first time, our study illustrate poplar pollen dispersal by the wind by the use of pollen traps, and the pollen was detected up to a distance of 540 m, the maximum distance analyzed. Nevertheless, genetic data reported in "Section 2.2 Breeding evaluation" showed that pollen of poplar can arrive up to a distance of 2 km. For oilseed rape, there are few studies that take into account only the individual plant pollen dispersal by the wind (Lavigne et al., 1998; Klein et al., 2006). In our study we found that pollen of oilseed rape and of sunflower arrives at 34 m and at 19 m, respectively. In addition, genetic data on pollen take from the hive indicated that the insect pollinator, *Apis mellifera*, can transport the pollen of sunflower up to a distance of 1 km.
The range of pollen dispersal needs to be further investigated in order to limit out-crossing by proposing containment measures also for future GM species that could be posed in commercialization.
Using the data provided by pollen flow, it was possible to simulate pollen dispersal as reported in "Chapter 5 geographical information systems for environmental risk assessment and GMO monitoring".

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Chapter 2

Pollen flow and breeding evaluation

2.2 - Breeding Evaluation

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Introduction

Gene flow between crops and wild species relatives has been occurring for thousands of years (Hancock et al. 1996, Ellstrand et al. 1999), but scientific attention on gene flow from crops to wild relatives and other crop populations is more recent, stimulated by concerns about the movement of transgenes (Snow and Morán-Palma 1997, Hall et al. 2000, Ellstrand 2002). Regardless of whether transgenes are involved, the consequences of gene flow from crops can be problematic. Crops genes may replace wild genes, reducing the genetic diversity of wild populations. Crop genes may also flow to other crop varieties or land races, contaminating the recipient seed pools. Whether this genetic contamination is called “genetic pollution” or “adventitious presence”, it can have undesired consequences, reducing seed quality (Friesen et al. 2003), threatening food safety (NRC 2004) and organic food production, or harming indigenous cultures [North American Free Trade Agreement– Commission for Environmental Cooperation (NAFTA–CEC) 2004]. If the resulting hybrids have lower fitness than their wild parents, the wild populations may shrink, threatening the survival of the wild population (Ellstrand and Elam 1993, Levin et al. 1996). Alternatively, if the resulting hybrids have higher fitness than their wild parents, they may become invasive (Tiedje et al. 1989), replacing the wild population and other species in agricultural and natural areas. Domesticated plants represent lineages that diverged from their progenitors no more than a few thousand generations ago. There is no reason to assume that reproductive isolation should be absolute (Ellstrand et al. 1999). Whether the evidence is reviewed on a regional basis or a crop-by-crop basis, it is clear that spontaneous hybridization and introgression of genes from domesticated plants to wild relatives is a common characteristic of domesticated plant taxa (Ellstrand et al. 1999). A major issue for plant genetic engineering is the extent to which transgenes will escape

from cultivation and cause negative impacts in wild ecosystems (Rogers et al. 1995, Wolfenbarger and Phifer 2000). Gene flow to wild relatives occurs for nearly all crops in places where they are grown (Ellstrand et al.1999). However, it is of particular concern for forest trees because they are virtually undomesticated (Bradshaw and Strauss 2001), they have the potential for spatially extensive gene flow (Hamrick et al. 1992, Slavov et al. 2002), and they can have large effects on ecosystem processes and biological diversity when they are the dominant life form (Wells et al. 1986). The ecological impacts of transgenic trees will primarily depend on the traits conferred by the transgene and the environment in which the trees are grown (Mullin and Bertrand 1998, James et al. 1998). Risk assessment therefore requires detailed consideration of the specific ecological consequences of individual transgenes in different settings. However, gene flow is a prerequisite for most ecological impacts outside of plantations, so baseline estimates of introgression will apply to most environmental risk assessments for transgenic trees (Ellstrand 2001, Muir 2001). The poplar has become a model tree species in genetic engineering as it can easily be transformed and clonally propagated and has a small genome size (Boerjan 2005). Tree growth, agronomic traits, and timber quality can be improved through genetic engineering (Pullman et al. 1998), thereby avoiding the long reproductive cycles of conventional breeding (Mathews and Campbell 2000). The potential environmental hazards linked to GM trees differ from those associated with transgenic crop plants at both spatial and temporal scales (van Frankenhuyzen and Beardmore 2004) because trees are long-lived perennials, unlike annual crop plants.

The evaluation of breeding was focused on two species: oilseed rape and poplar. The choice was conditioned by the presence of wild relatives with the same periods of phenological development for these two species in the study areas of the DEMETRA project.

Results and discussion

1. Breeding evaluation in poplar

In the protected area of Regional Park of Migliarino – San Rossore – Massaciuccoli two permanent study Areas 1 and 2, 8 Km faraway, are detected and placed in different ecosystems (Chapter 1).

Fig. 1. Study Area 1 (Massaciuccoli Lake).

The Study Area 1 is a wetland habitat, where single trees and small groups of poplar are scattered along the shores of the Massaciuccoli Lake (Fig. 1). In relation to the herbaceous stratum, the observations made so far show some variability in the distribution, probably due to the micro topological characteristics of the site. Sedges and reeds communities growing in the area have to be referred to the class *Phragmito-Magnocaricetea* Klika. This site is characterized by many rare or

threatened species, such as: *Periploca graeca* L., *Leucojum aestivum* L., *Hibiscus palustris* L. (Chapter 3). A total of 32 poplars were identified and their position was collected by GPS. Plant tissue was taken from each poplar for subsequent laboratory analysis.

The Study Area 2 is a naturally-originated mixed forest stand, in proximity of Serchio river, where the prevailing tree species are poplar, *Fraxinus angustifolia* and *Alnus glutinosa*, the latter in the lower strata (Chapter 1). According to the vegetation analysis, the woods with poplar can be referred to *Carici remotae-Fraxinetum oxycarpae* Pedrotti (Chapter 3). In this area within an experimental plot, 7,000 m^2 large, all poplar trees (30 individuals) were identified and their position was collected by GPS (Fig. 2). Even in this case, plant tissue was taken from each poplar for subsequent laboratory analysis. The poplars in the Study Area 2 are morphologically classified as belonging to the *Populus alba* (white poplar) species and hybrid *P. x canescens* (gray poplar). *P. alba* and *P. tremula*, parental of the hybrid *P. x canescens*, are two ecologically divergent species that hybridize frequently in Europe. In addition, the species of poplars sampled was identified by sequence analysis of the *trn*L-*trn*F cpDNA (chloroplast DNA) region (Table 1.).

Fig. 2. Study Area 2 (Serchio river).

Table 1. *Variable sites into trnL-trnF cpDNA region of considered poplar species.*

	Variable Sites[a]		
	198	279	478
P. alba	T	A	A
P. x canescens	T	A	A
P. tremula	T	A	G
P. nigra	T	A	A
P. deltoides	G	G	A
P. x euramericana	G	G	A

[a] Primers used as reported in Taberlet et al. 1998; amplification conditions and alignment sequences used as reported in Paffetti et al. 2007.

Fig. 3. Study Area 2 (Serchio river). In yellow color are reported the position of gray poplar.

Moreover, the poplars were genotyped using 10 nuclear microsatellites (nSSR) loci[1]. The nuclear microsatellites present unique allelic variant for each species. The results show that in the study plot 6 trees belong to gray poplar, including 5 females and 1 male, and 23 males and 1 female are white poplars (Fig. 3 and Table 2).

The cpDNA[2] haplotype variant is identical for all individuals, as the mother of the hybrid *P. x canescens* is *Populus alba*. The values of genetic diversity, given

here only as an average of the data obtained by 10 microsatellite loci, appear to be low (Table 3), but consistent with the data previously reported in literature for natural populations of the two species (Lexer et al. 2005).

Table 2. Number (ID), sex and molecular identification by cpDNA haplotype and nSSR genotyping of each poplar tree considered in the Study Area 2.

ID poplar tree	*Sex*	*cpDNA haplotype*	*nSSR genotyping*
P1, P2, P3, P4, P6	♀	TAA	*P. x canescens*
P10	♂	TAA	*P. x canescens*
P7, P8, P9, P11, P12, P13, P14, P15, P16, P17, P18, P19, P20, P21, P22, P23, P24, P25, P26, P27, P28, P29, P30	♂	TAA	*P. alba*
P5	♀	TAA	*P. alba*

Table 3. Genetic variability estimates. Number of alleles (N), number of rare alleles (N_{rare}), expected heterozygosity (H_e) and fixation index (F_{IS}).

	P. x canescens				*P. alba*			
	N	H_e	N_{rare}	F_{IS}	N	H_e	N_{rare}	F_{IS}
Mean	1.4	0.174	0.00	0.002	3.0	0.350	0.25	-0.001

We inferred spatial population structure using a Bayesian Monte Carlo Markov Chains method implemented in the Geneland package (Guillot et al. 2005) under the R Language. The results show that the population is divided into four clusters (Fig. 4). The clusters are genetically isolated, as indicated by the maps of posterior probability, but each cluster does not seem to be composed of trees related to each other. The cluster

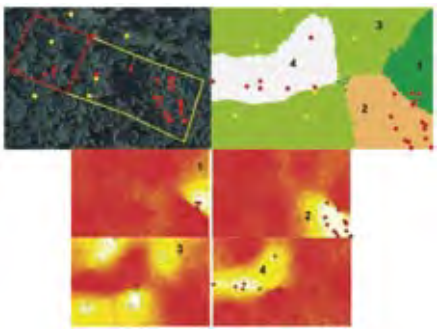

Fig. 4. Spatial organization into four clusters and maps of posterior probability of each cluster.

Fig. 5. Study Area 1 (Massaciuccoli Lake). Yellow dots represent the positions of gray poplars, red dots of white poplars and green dots of black poplars.

[1] Primers and amplification conditions used as reported in Tuskan et al. 2004 and Smulders et al. 2001.
[2] In poplar, the chloroplast in the chloroplast is inherited from the mother.

indicated by the number 3, is exclusively composed of *P. x canescens* trees. This cluster appears genetically more isolated from others.
In addition to the two poplar species, *P. alba* and *P. x canescens*, present in the Study Area 2, in the Study Area 1 trees attributable to the *P. nigra* (black poplar) morphology were identified. This was confirmed by the analysis of sequence data and genotyping (Fig. 5 and Table 4). As expected, the cpDNA haplotype of *P. nigra* is identical to *P. alba* haplotype, and black poplar presents the specific microsatellite allelic variants for the species. The individuals for this species show the higher levels of genetic diversity among all three species (Table 5).

Table 4. Number (ID), sex and molecular identification by cpDNA haplotype and nSSR genotyping of each poplar tree considered in the Study Area 1.

ID poplar tree	*Sex*	*cpDNA haplotype*	*nSSR genotyping*
PM1, PM2, PM3, PM5, PM6, PM10, PM17, PM20, PM29, PM30, PM31	♂	TAA	*P. alba*
PM12, PM23, PM25, PM27	♀	TAA	*P. alba*
PM4, PM11, PM13, PM16, PM18, PM19, PM21, PM24, PM28	♂	TAA	*P. x canescens*
PM22, PM26, PM32	♀	TAA	*P. x canescens*
PM8, PM15	♀	TAA	*P. nigra*
PM7, PM9, PM14	♀	TTA	*P. nigra*

Table 5. Genetic variability estimates. Number of alleles (N), number of rare alleles (N_{rare}), expected heterozygosity (H_e) and fixation index (F_{IS}).

	P. alba				*P. x canescens*				*P. nigra*			
	N	H_e	N_{rare}	F_{IS}	N	H_e	N_{rare}	F_{IS}	N	H_e	N_{rare}	F_{IS}
Mean	3.6	0.445	0.22	0.012	3.6	0.550	0.17	0.011	3.3	0.565	0.00	0.003

The spatial structure population has a division of population into three clusters. In this case, as evidenced by the maps of posterior probability and the values of F_{ST}, only the cluster 1, comprising the *P. nigra* tree species, is genetically isolated from the others. On the contrary, gene flow is evident between the other two clusters consisting of *P. alba* and *P. x canescens* trees. The different clusters are constituted by unrelated individuals between them (Fig. 6).
Both the Study Area are adjacent to poplar plantations. In particular, the Study Area 2 is adjacent to "Triplo" multiclonal plantation and to "Onda" multiclonal plantation. The "Triplo" is a hybrid of *Populus deltoides* (mother) and *Populus nigra* (father), defined *P. x euramericana*. While "Onda" is a *P. deltoides* selection. The Study Area 1 is adjacent to

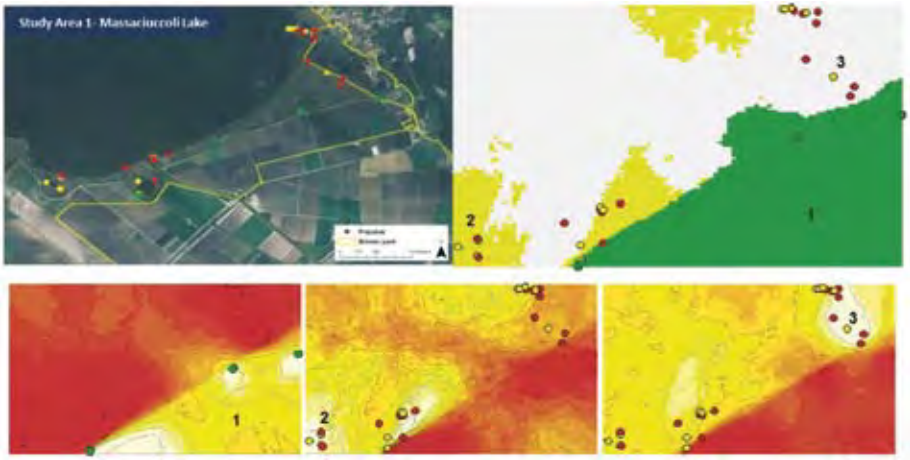

Fig. 6. Spatial organisation into three clusters and maps of posterior probability of each cluster.

poplar multiclonal plantation, a *P. deltoides* selection. The sequence analysis shows that the mother of cultivated varieties is *P. deltoides* and genotyping allows the identification of each cultivated variety (Table 6).

After analyzing the species phenology, in particular, after confirming the correspondence of pollen production period from plantations and the receptivity ovary period of female trees present in natural populations, the genotyping of the offspring, germinated seeds, was performed by the same nuclear microsatellites loci used for identification.

Table 6. Number (ID), sex and molecular identification by cpDNA haplotype and nSSR genotyping of each poplar plantation.

ID poplar tree	*Sex*	*cpDNA haplotype*	*nSSR genotyping*
"Onda" plantation (Study Area 2)	♀	GGA	*P. deltoides*
"Triplo" plantation (Study Area 2)	♀	GGA	*P. x euramericana*
Poplar plantation (Study Area 1)	♀	GGA	*P. deltoides*

We conducted paternity assignment using all ten analysed nSSR loci by standard maximum-likelihood methods implemented in CERVUS 3.0 (Marshall et al. 1998). Critical likelihood values (LOD-scores) yielding 95% confidence in assignments were obtained using simulations.

In Study Area 1, the crossings of PM 11 (gray poplar), PM 20 and PM 23 (white poplar) mother trees were identified (Fig. 7). The crossings involved male individuals belonging to both species, but, as expected, hybrids with black poplar were not detected. In Study Area 2, the crossings of P 1, P 2 and P 6 mother trees belonging to the species gray poplar with individuals of white poplar were identified. Only the P 2 mother tree produced offspring with the P 10 male tree of the same species. The P 5 mother tree, *P. alba,* produced offspring only with individuals of its own species (Fig. 8).

The P 3 mother tree, *P. x canescens*, produced offspring with individuals of *P. alba* species (Fig. 8). But unexpectedly the hybridization occurred between this tree and "Triplo"

Fig. 7. The crossings identified in the Study Area 1.

Fig. 8. The crossings identified in the Study Area 2.

plantation. The analysis of forest structural data (diameter and height trees) showed, that P 3 is a large tree which have not barriers to the pollen flow from the plantation due to competition with other crowns. Furthermore, thanks to the analysis of the wind direction and speed, it was possible to find that during the pollen diffusion and ovary receptivity the direction east-northeast to west-southwest of the wind favored pollen dispersion from F1.1 tree ("Triplo") to the P3 tree (Fig. 9).

This work confirms the presence of *P. x canescens* in naturally-originated stand. In addition, it was possible to identify the presence of the hybrid in the Massaciuccoli Lake. In the mixed forest the *P. x canescens* subpopulation appears spatially genetically isolated. While, there is not the same situation on the Lake. The *P. alba* and *P. x canescens* groups, in this case, present higher level of genetic variability and an evident gene flow. This is probably related to different environmental conditions. The detection of breeding, between the tree in Study Area 2 and the plantation, suggests the occurrence of a possible genetic exchange among a natural population and plantation which has to be carefully considered

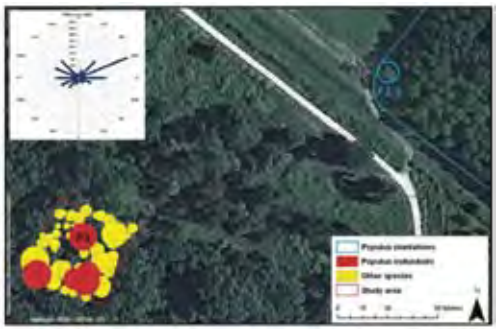

Fig. 9. Crossing between P 3 and F 1.1. poplar.

when poplar plantation are made close to natural environment in which wild relatives are present. The interspecific incompatibility in *Populus* has been extensively studied with the aim of obtaining hybrids for forest tree breeding programs. Hybrids are freely obtained between species within a section, but not between section. However, in the literature several examples reported the unilateral hybridization phenomena. For example Ronald (1982) reported that the cross of white poplar-aspen (*P. tremula*) hybrid female partner to *P. deltoides*, to *P. nigra* and to *P. x euramericana* produced a quantity of viable seeds and seedling.

2. Breeding evaluation in oilseed rape

Some wild plant species belonging to Brassicaceae have been selected in the protected areas of Regional Park of Migliarino – San Rossore – Massaciuccoli, to evaluate possible hybridization and gene flow. The choice of Brassicaceae has been done considering the pollinating fauna and the flowering period. *Sinapis arvensis* (Fig. 10) was consider as one possible candidate to hybridize with *Brassica napus* L. var. *oleifera* Del. (oilseed rape) (Chapter 3). Due to the close genomic relationship between these taxa, nSSR primers designed for different Brassicaceae species were tested to amplify both in *Brassica napus* and *Sinapis arvensis*. Therefore we looked for *S. arvensis* (field mustard) populations and oilseed *B. napus* cultivars which would hybridize in the field. To verify possible hybridization between *Brassica napus* and *Sinapis arvensis* 41 nuclear microsatellite markers were tested. These markers, derived from literature (Lowe et al. 2002, 2004, Szewc-Mc Fadden et al. 1996, Lagercrantz et al.1993) were designed on different species of Brassicaceae and were selected on the basis of their transferability. Only 10 primer pairs showed amplification in both *Brassica napus* and *Sinapis arvensis* samples. Among them, 2 primer pairs distinguished different alleles between *B. napus* and *S. arvensis*. In order to verify if hybridization occurs in the field, a sampling of field mustard (potential mothers) and oilseed rape (potential fathers) plants was performed (Fig. 10). Ten plants of field mustard were randomly chosen as mothers and georeferenced. All potential fathers of oilseed rape (442 individuals in total) were sampled within 3 m radius away from field mustard (mother) and their distance from mother as well as the

Fig. 10. Sinapis arvensis L.

Fig. 11. Experimental process using to breeding evaluation between oilseed rape and field mustard.

cardinal direction were registered (Figure 10). About 100 seeds per mother were collected by "Arasystem" (Betatech bvba) traps, soaked with a KNO_3 solution and posed in Petri dishes to germinate (Fig. 11).

The plants of wild mustard (indicated by the red color in Fig. 12) have allelic variants that distinguish them from two varieties of rapeseed cultivation (CV1 and CV2 indicated with black and yellow color in Fig. 12, respectively).

Furthermore, these cultivars can be distinguished by different allelic variants. It was thus possible to determine that most of the seeds appear to be the product of self-fertilization of *Sinapis arvensis*, as expected. But a smaller percentage of the seeds turns out to be the product of fertilization between different individuals of wild mustard, and between plants of wild mustard and rapeseed. In particular, the plant of wild mustard indicated with the number 3 and the plant indicated with the number 10 have produced hybrid offspring with rape plants of CV2.

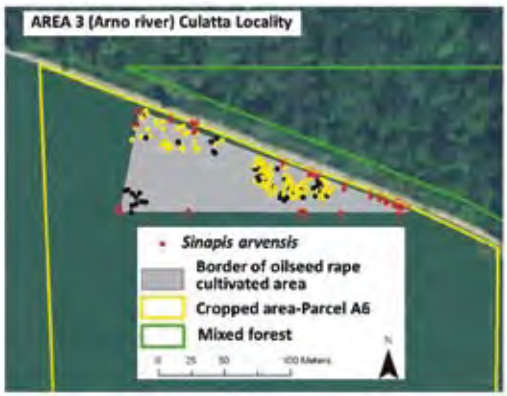

Fig. 12. Sinapis arvensis plants (mother) and oilseed rape (father): CV1 (black dots) and CV2 (yellow dots).

If GM oilseed rape is to be grown, the possibility of his modified trait being transferred to *S. arvensis* needs serious consideration as the species are widespread.

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Chapter 3

Assessment of local biodiversity

3.1 - Plant diversity (floristic and vegetation analysis)

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The analysis of plant biodiversity within the study area has regarded the census of the plant species (vascular plants), their phenology as well as the identification of those species susceptible to breeding with the cultivated target species, and the identification of the plant communities and their relationships with the main environmental factors.

Material and Methods

Within the study area specific study sites have been identified and recognized on the basis of the main ecological features (environment). The study sites are: natural poplar forest, mixed forest, pine forest, "Serchio" fallow lands (parcel S21), "Culatta" cropped area (parcel A6), "Lame" wet meadows and Massaciuccoli Lake wetlands.
Within each of these sites, permanent sample plots have been selected, variable in number and size, according to extent and type of vegetation. The main sample plots are 1000 m^2 squares divided, where possible, in sub plots of 100 m^2 and 10 m^2, according to the nested-squares scheme proposed by Dengler (2009) to assess plant diversity patterns of individual plots of interest, along environmental gradients, within specific vegetation types, or for landscape sectors. For the assessment of plant biodiversity in forest environments, a variable number of 1000 m^2 plots (and nested sub-plots) have been used, according to the extent of the area under examination. In the case of the "Lame" wet meadows, 10 m^2 plots have been chosen, because of the particular topography of the site. In the case of the Massaciuccoli wetlands, the setting of permanent plots was not feasible, due to the particular morphology of the site, and then the plant biodiversity assessment was carried out by means of samplings randomly performed in the study site.

The sampling of the vascular flora was carried out from the month of March (for the early flowering) until the month of November (for the late blooms and for the collection of the last phenological stages). On the basis of the collected specimens, a database has been created. For each *taxa* have been reported: botanical family, life form (Raunkiaer, 1934), chorological group (Pignatti, 1982; to avoid fragmentation into chorological groups and

subgroups, the chorotypes were properly merged in main groups) and bio-ecological index, according to Pignatti et al. (1996). For the nomenclature, Conti et al. (2005) and amendments (2007) have been followed.

On the basis of this data base, for each study site have been evaluated: a) species distribution in botanical families; b) distribution for biological types; c) chorological spectrum, highlighting the geographical distribution.

The data deriving from the application of the bio-ecological index of Pignatti-Ellenberg have been processed. The bio-ecological index is a valid system to correlate the environmental conditions with the presence of plant species or plant communities, providing a range of information on the ecological factors characterizing a specific area (Ellenberg, 1974; Pignatti et al., 1998). To each species is assigned a value for each of six environmental factors, according to precise numerical scales:

- Light radiation (L) : 1 to 9;
- Temperature (T): 1 to 9;
- Climate continentality (C): 1 to 9;
- Moisture (U): 1 to 12;
- Soil reaction (R): 1 to 9;
- Nutrients availability (N): 1 to 9;
- Salinity (S): 1 to 3.

The values can then be processed in a radar-graph called eco-gram (Pignatti et al., 1996).

The presence of threatened/rare species has been detected by following the national and regional red lists (Conti et al. 1992; 1997)

The vegetation analysis has been carried out by following the Zürich-Montpellier phytosociological method (Braun-Blanquet, 1964). Phytosociological relevés have been performed within the selected plots and sub-plots, in the period March–October 2010 and mapped using a GPS. The plot size varies from 1000 m^2 to 10 m^2, depending on vegetation type and microtopography.
The phytosociological relevés have been arranged in different matrices: woody vegetation, herbaceous vegetation of wet meadows and herbaceous communities of fallow lands. For each dataset, similarity analyses have been carried out by using the SYN-TAX 2000 software (Podani 2001). Original Braun-Blanquet sampling scale has been transformed into the ordinal scale according to Van der Maarel (1979). A hierarchic classification method (UPGMA) was performed. Dissimilarity of the relevés was measured using the Euclidean distance. The ordination of the data-sets was performed using the PC-ORD 4.34 software. In the ordination analyses the Nonmetric Multidimensional Scaling (NMS) has been ran,

based on the Euclidean distance.
The syntaxa have been assigned according to the classification of Rivas-Martinez et al (2001; 2002).

In order to identify, among the surveyed wild species, those susceptible to potential breeding, a pool of target species belonging to the family Brassicaceae was selected (namely, the Brassicaceae recorded in the study area "Culatta") and their phenology has been consistently observed. Starting from March 2010 and throughout the growing season, phenological data for the identified target species have been recorded, by a monthly basis, according to the BBCH protocols (Biologische Bundesanstalt, Bundessortenamt, Chemische Industrie) (Schwartz, 2003).

Floristic analysis: distribution for botanical family, life form and chorological group

During the period of observation, an overall amount of 307 plant taxa, distributed into 57 families, have been recorded. *Poaceae* (17.6%), *Asteraceae* (12%) and *Cyperaceae* (8.5%) are the prevailing.
Comparing the species distribution in the different study areas (Fig. 1-5), *Poaceae* comes out as the most represented family in all the considered environments, with the highest value (42%) recorded in the pine forest.
The only exception is the forest with natural poplar, where *Cyperaceae* results to be the most represented family, probably due to the prolonged flooding period. In general, all the humid environments such as wet meadows ("Lame"), Massaciuccoli lake wetlands and hygrophilous forests, are characterized by a large presence of species belonging to the family *Cyperaceae*.
Other families with significant percentages are *Asteraceae* and *Fabaceae*. A fairy high rate of taxa belonging to the *Ranunculaceae* family occurs in the wet meadows.

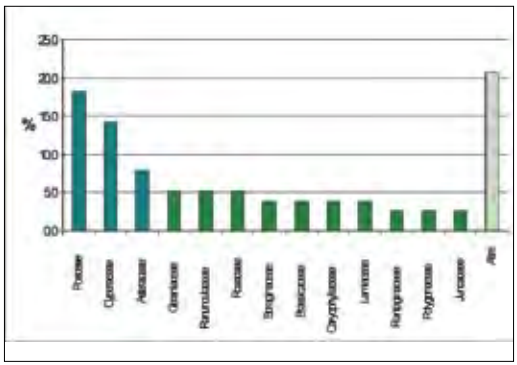

Fig. 1. Distribution for botanical family of the species collected in the mixed wood

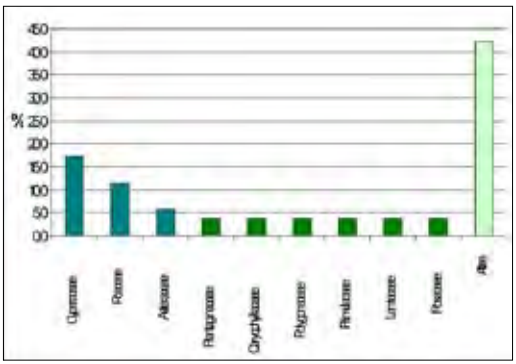

Fig. 2. Distribution for botanical family of the species collected in the wood with poplar

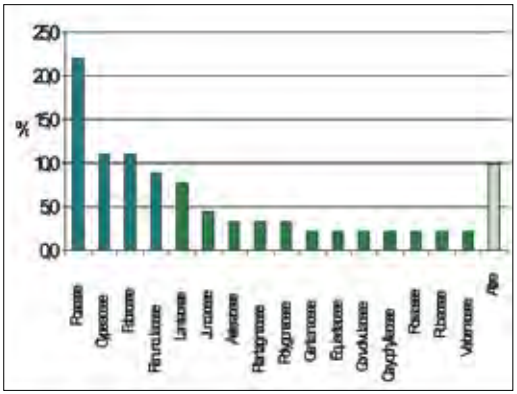

Fig. 3. Distribution for botanical family of the species collected in the wet meadows (Lame)

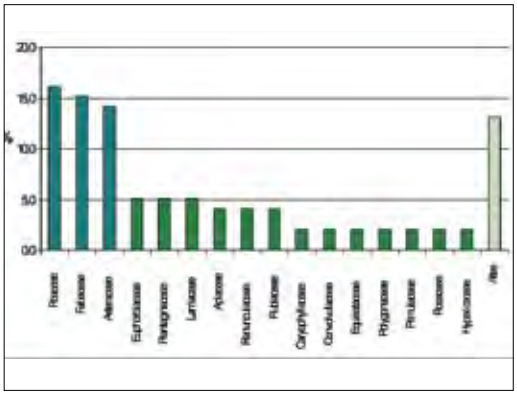

Fig. 4. Distribution for botanical family of the species collected in the fallow lands (Serchio)

As regards the species distribution according the life forms (Fig. 6), Hemicryptophytes (H) are the prevailing, with a total percentage of 44.4%, as expected in humid environments mainly represented by hygrophilous woods and grasslands.
Therophytes (T) have an important percentage (32%), due to their massive presence in semi-natural environment (fallow lands) subject to intensive anthropic pressures. Geophytes (G) and Phanerophytes (P) have also significant values.
It's worth noting that life forms specifically linked to wet environments (Helophytes (He) and Hydrophytes (I)), although present with a fair amount of taxa, rarely reach significant percentages.

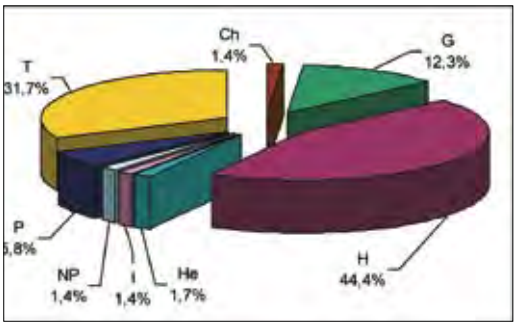

Fig. 6. Distribution of the species collected in the study areas for life forms

The chorological spectrum (Fig. 7) shows a clear predominance of cosmopolite and subcosmopolite types, with very high values (24%).
These entities are generally related to environments with very high anthropic pressure. The eurimediterranean type shows a quite significant value (16%). Other chorotypes have percentages of about 7-8% and are characterized by large distribution area (Circumboreal, Eurasian, European, Paleotemperate).

The presence of the Mediterranean element has scarce significance.

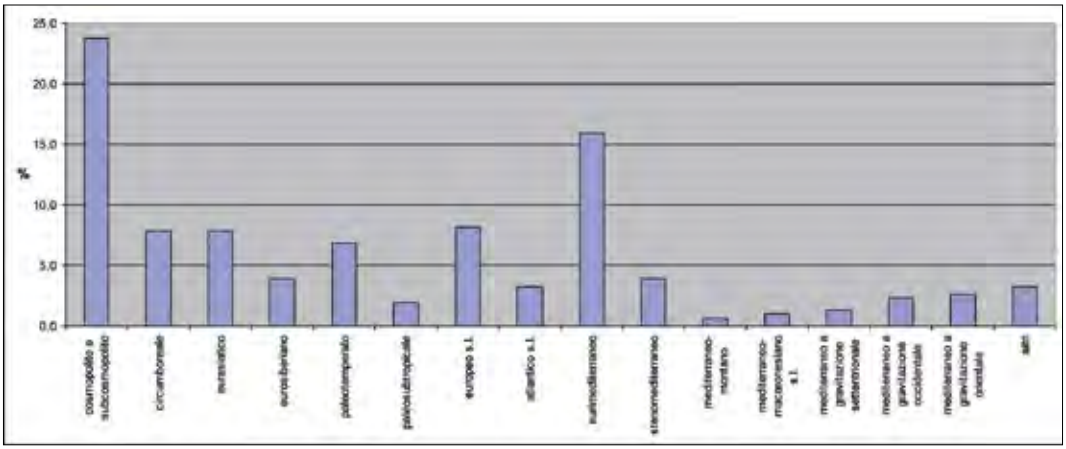

Fig. 7. Distribution of the species collected in the study areas for chorological type

The Pignatti-Ellenberg eco-grams provide useful information about the ecology of the study areas.

As for the light radiation (L), grasslands show high values, revealing a significant component of plant species that prefer direct and constant sunlight, as confirmed by the individual eco-grams of these areas (fallow lands, wet meadows and wetlands).

The hygrophilous forests, namely the mixed forest (Fig. 8 -A) and the forest with natural poplar (Fig. 8 - B), show instead the lowest values of L, due to the presence of a large amount of sciaphilous species.

The pine forest (Fig. 8 - C) differs from all the other study areas for the highest values of L and T, due to the presence of more thermophilic species.

The soil moisture (U) reaches very high values in hygrophilous forests, wet meadows and wetlands confirming, in these areas, the prevalence of taxa that need soil well supplied with water and enduring long flooding periods.

The pine forest eco-gram shows the lowest U value; this environment is characterized by soils with high permeability and by the presence of species tolerating a certain degree of drought in summer.

The availability of nutrients (N) points out the presence of species with optimal growth on well-humified soils provided in organic matter and nutrients.

These values are slightly higher in the fallow lands (Fig. 8 – D and E) and, surprisingly, in the hygrophilous forests with poplar (Fig. 8 - B), and in the lake wetlands (Fig. 8 – F and G). In the last case, the high N value is due to the particular position of some study plots, located in proximity of the boundaries between wetlands and cultivated areas. In the pine forest there is a strong presence of plants preferring oligotrophic soils.

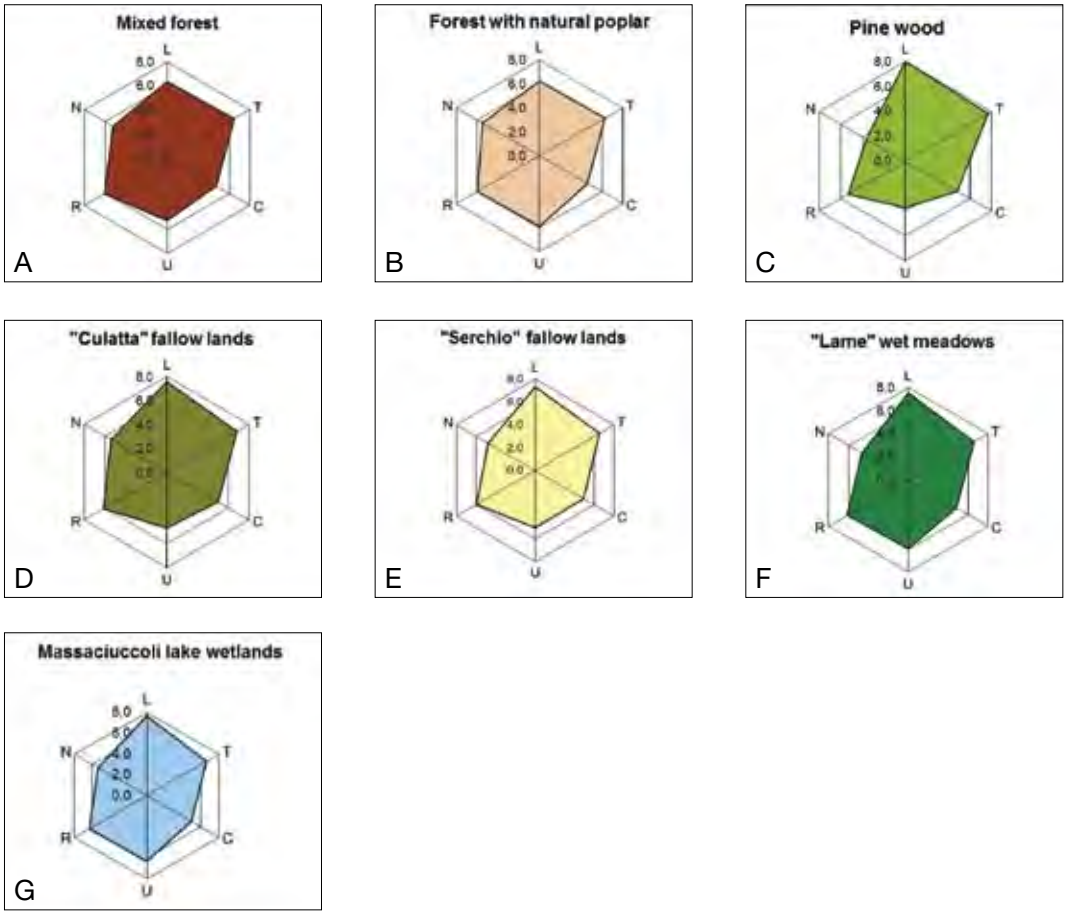

Fig. 8. Ecograms (Pignatti-Ellenberg) for the study sites: A) mixed deciduous forest; B) deciduous forest with natural poplar; C) pine wood; D) "Culatta" fallow lands (cropped area); E) "Serchio"fallow lands (uncropped area); F) "Lame"wet meadows; G) Massaciuccoli lake wetlands.

Floristic analysis: rare and threatened species in the study areas

A significant amount of rare and threatened species, as belonging to national and regional red lists, or to the CITES lists or to the Annex II of the Habitat Directive (92/43 EEC Directive), has been detected.

The large part has been found in the area of the Massaciuccoli lake: *Periploca graeca* L. (Fig. 9) and *Hibiscus palustris* L. (Fig. 10) [vulnerable (VU) at national level]; *Hydrocotyle vulgaris* L.[endangered (EN) at national level] and *Anagallis tenella* (L.) L. [critically endangered (CR) at national level]. In addition, a rich contingent of plant species threatened at regional level has been found: *Leucojum aestivum* L. subsp. *aestivum* [critically endangered (CR)] and *Cladium mariscus* (L.) Pohl [low risk (LR)], *Euphorbia palustris* L. [vulnerable

(VU)], *Carex panicea* L. [vulnerable (VU)], *Carex vesicaria* L. [vulnerable (VU)], *Oenanthe lachenalii* Gmelin [vulnerable (VU)] and *Thelypteris palustris* Schott [vulnerable (VU)]. All the mentioned species are strongly affected by alterations of the ecological factors directly influencing the wetland environment and, in particular, those related to water quality and regime.

As far as the other environments/study sites, only few plant species of conservation interest have been found: *Ranunculus flammula* L., in the wet meadows of the site "Lame", vulnerable (VU) at national level; *Carex panicea* L., growing in the herbaceous layer of the mixed wood, vulnerable (VU) at regional level; *Ranunculus ophioglossifolius* Vill vulnerable (VU) at national level and found both in the "Lame" wet meadows and in the mixed wood.

Fig. 9. Periploca graeca

Fig. 10. Hibiscus palustris

Floristic analysis: phenology and potential breeding

In order to identify the wild plant species susceptible to potential breeding with the cultivated oilseed rape (*Brassica napus* L. var. *oleifera* D.C.), a group of species belonging to the *Brassicaceae* family and recorded in the study area "Culatta" has been consistently observed following their phenological phases, starting from March 2010 and throughout the growing season. The recorded and surveyed species are: *Sinapis arvensis* L., *Cardamine hirsuta* L. (Fig. 11), *Capsella bursa pastoris* (L.) Medicus (Fig. 12), *Cardamine pratensis* L. and *Alliaria petiolata* (Bieb.)Cavara et Grande (Fig. 13). *Sinapis arvensis* and *Capsella bursa-pastoris* have been recorded in the fallow lands surroundings the cultivated areas, *Cardamine pratensis* and *Alliaria petiolata*, both mesophilous species, have been collected in the herbaceous layer of the mixed forests, while *Cardamine hirsuta* has been found in both the environments.

Throughout the growing season, phenological data for the identified species have been recorded by a monthly basis. In order to make easier the data interpretation, a specific symbology has been used. In table 1 the main phenological stages of the observed species have been reported.

Sinapis arvensis has been found to be the best candidate for potential breeding with the

cultivated oilseed rape, for distribution, frequency and for the more or less simoultaneous (and long) flowering period. The high potential of breeding is confirmed by literature data, for the marked IHP (introgressive hybridization propensity; Devos et al., 2009) and the high SC (sexual compatibily; Letorneau et al., 2003).

Fig. 11. Cardamine hirsuta

Fig. 12. Capsella bursa-pastoris

Fig. 13. Alliaria petiolata

Table. 1. Phenology of Brassicaceae in the study area "Culatta". "F" is fallow lands; "MW" is mixed wood.

Species	Family	Environment	March	April	May	June	July	August	Septemb
Sinapis arvensis L.	Brassicaceae	F	□	□	□ •	□ •	□ •	•	
Cardamine hirsuta L.	Brassicaceae	F/MW	□	•					
Capsella bursa-pastoris (L.)Medicus	Brassicaceae	F	□	•					
Cardamine pratensis L.	Brassicaceae	MW		□	•				
Alliaria petiolata (Bieb.)Cavara et Grande	Brassicaceae	MW			□	•			

□ = full flowering
• = ripe fruits
= seed dispersion

Vegetation analysis: plant communities

Massaciuccoli lake

As already highlighted by Tomei et al. (1997), the plant communities growing in the wetlands of the Massaciuccoli lake are divided in the following types:

a) vegetation dominated by hydrophytes (i.e. aquatic plants, entirely or mostly submerged in water, with the exception of floating leaves; in this group are included the plant communities with *Myriophyllum verticilaltum* L. and those with *Ceratophyllum demersum* L., in eutrophic waters; *Potamogeton pectinatus* L. plant communities and, in oligotrophic waters, *Nymphaea alba* communities) - this vegetation can be referred to the habitat 3150 (Natural euthrophic lakes with Magnopotamion or Hydrocharition-type vegetation), according to the Directive 92/43 EEC;

b) vegetation dominated by helophytes (i.e., "amphibious" plants with the bottom part submerged for most of the year and with aerial leaves; this group includes the common

reed and many species belonging to *Juncacee* and *Cyperaceae*, typical of marshy environments);
c) wet meadows (perennial hygrophilous grasslands) - habitat 6420 (Mediterranean tall humid herb grasslands of the Molinio-Holoschoenion)
Group "b" (helophytes) includes the plant communities with *Phragmites australis* (Cav.) Trin. and those with *Cladium mariscus* (L.) Pohl. *Cladium mariscus* plant communities refer to the association *Cladietum marisci* (Allorge 1922) Zobrist 1935, corresponding to the priority habitat 7210 (Calcareous fens with *Cladium mariscus* and species of the *Caricion davallianae*) according to Directive 92/43 EEC, and have fairly limited extent respect to the reed beds. *Phragmites australis* is an invasive species that tends to encroach upon the *Cladium mariscus* communities and, over time, to spread and replace them. The reed beds are referred to the association *Phragmitetum communis* (Koch 1926) Schmale 1939 (Fig. 14). In some marginal areas, along the boundaries between the dams and the reed beds, *Schoenoplectus lacustris* (L.) Palla populations occur (referred to the association *Scirpetum lacustris* Schmale 1939).
Moving to the inland area, subject to grazing and other human activities, fallow lands characterized by sub-nitrophilous perennial herbaceous communities (*Artemisietea vulgaris* Lohmeyer, Preising & Tüxen ex von Rochow 1951 class) tend to prevail.
As regards the analysis of specific sites of investigation, many of them fall within the helophytes communities and, precisely, within the *Phragmites australis* and the *Schoenoplectus lacustris* communities. Two sites lie in transition areas characterized by shrub communities with *Rubus ulmifolius* Schott and *Calystegia sepium* (L.) R. Br. and by the presence of the rare *Periploca graeca* L. Finally, two sites fall within the herbaceous communities, dominated by annual and perennial synanthropic and sub-nitrophilous species of fallow lands.

Fig. 14. Massaciuccoli lake, Phragmitetum communis

"Lame" wet meadows

The whole study area of S. Rossore is characterized by a large extent of wetlands and wet meadows. In the inland these environments are characterized by fresh waters whilst, near the coastline, brackish and salt waters (and then, halophilous and sub-halophilous plant communities) tend to prevail. Due to this variability in water quality and edaphic factors, salt marshes and wet meadows host a wide range of vegetation types (Tomei et al., 2004). The wet meadows of the site "Lame" (Fig. 15), analyzed in this study, constitute a varied environment with an intricate mosaic of vegetation types related to different flooding period, soil moisture and other edaphic factors. This is well shown by the cluster analysis (UPGMA-Euclidean) which highlights three sub-clusters (A1, A2 and B), each of them

linked to a particular water regime (Fig. 20). The outcome of the cluster analysis is confirmed by the ordination diagram (Fig. 21), showing the relevés of wet meadows dispersed in three groups corresponding to the three main sub-cluster. Along the Axis 1, a clear gradient linked with soil moisture and flooding period is evidenced. The first group on the left of the graph (corresponding to the sub-cluster A2) is formed by those stands characterized by a long flooding period. The presence and abundance of *Eleocharis uniglumis* (Link) Schultes, *Cyperus longus* L., *Gratiola officinalis* L. (Fig. 16) lead us to refer the vegetation to the *Phragmito-Magnocaricetea* Klika in Klika & Novák 1941 class and to the *Magnocaricion elatae* Koch 1926 alliance (Fig. 17). The group in the central part of the graph (corresponding to the sub-cluster A1) is formed by most of the stands, characterized by a shorter flooding period and referable, on the basis of the floristic composition [*Carex distans* L., *C. divisa* Huds. (Fig. 18), *C. hirta* L. (Fig. 19), *Holcus lanatus* L., *Poa trivialis* L., *Ranunculus repens* L., *Juncus inflexus* L.], to the *Molinio-Arrhenateretea* Tüxen class and to the *Menho-Juncion inflexi* De Foucault 1984 alliance (*Plantaginetalia majoris* Tüxen & Preising in Tüxen 1950 order). The group on the right of the graph, with only two relevés (corresponding to the cluster B) is represented by the most xeric stands, located in the elevated parts of the wet meadows and characterized by very short or no flooding period and by the dominance of *Anthoxanthum odoratum* L. and *Gaudinia fragilis* (L.) Beauv.. In Fig. 22 a scheme of the main plant communities occurring in this site is provided.

Fig. 15. Wet meadows, study site "Lame"

These vegetation types can been referred to the habitat 6420 (Mediterranean tall humid grasslands of the *Molinio-Holoschoenion*), according to the Annex I of the Habitat Directive.

Fig. 16. Gratiola officinalis

Fig. 17. Stands with Eleocharis uniglumis (Magnocaricion elatae)

Fig. 18. Carex divisa

Fig. 19. Carex hirta

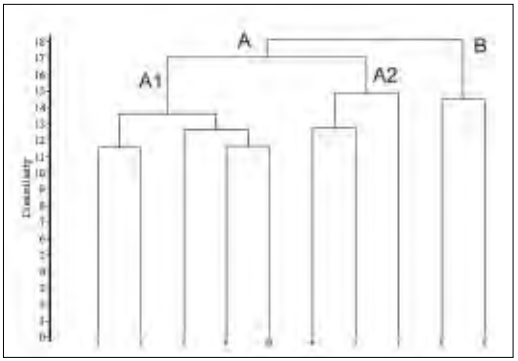

Fig. 20. Cluster analysis of vegetation stands of the site "Lame"

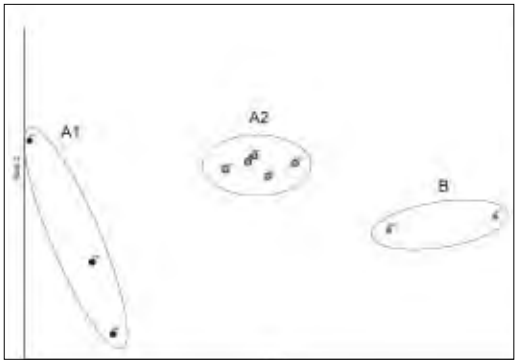

Fig. 21. Ordination diagram of vegetation stands of the site "Lame"; explanation is in the text

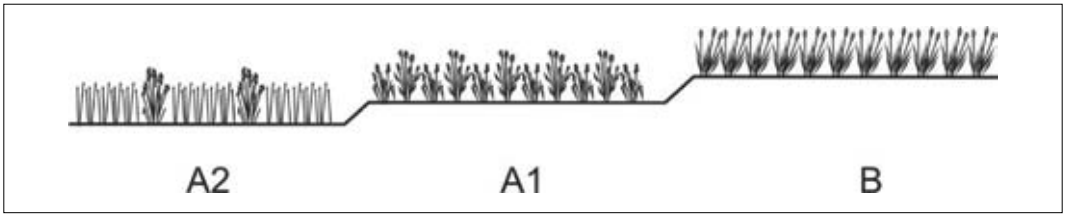

Fig. 22. An ideal schematic vegetation transect of the "Lame" wet meadows, from the parts subject to very prolonged flooding period (left), to the elevated parts subject to short (or no) flooding period (right). A2) hygrophilous communities with Eleocharis uniglumis and Cyperus longus (Phragmito-Magnocaricetea class); A1) meso-igrophilous communities with Carex sp.pl. (Molinio-Arrhenatheretea class); B) mesophilous communities with Anthoxanthum odoratum (Molinio-Arrhenatheretea class).

"Serchio" fallow lands

Tha fallow lands analyzed in the "Serchio" area are semi-natural areas in recovery after a long period of cultivation (poplar plantations). These fallows are surrounded by various environments such as other poplar plantations, wood fringes, pastures, wet meadows and drainage channels.

The high number of species recorded in the three plots of the "Serchio" fallows is related to the particular nature of the site. Here the typical species of fallow lands are mixed with

other originating from the surrounding environments (ecotone).
The cluster analysis (UPGMA - Euclidean distance) carried out on the nine sub-plots shows a clear distinction of two ecological groups (Fig. 23). Two main clusters are well differentiated: cluster (A) comprising the set of the plot S1 (sub-plots S1.1, S1.2, S1.3), characterized by a floristic component nitrophilous and rich in therophytes; cluster (B) comprising the sets of the plot S2 (sub-plots S2.1, S2.2, S2.3) and the plot S3 (S3.1, S3.2, S3.3) and characterized by a floristic component rich in mesic perennial and sub-nitrophilous species. The outcome of the cluster analysis is confirmed by the ordination diagram, showing the relevés of the fallow lands of the "Serchio" site dispersed in two groups (Fig. 24) corresponding to the two main clusters.
The group on the left of the graph (corresponding to the cluster B) is formed by those stands characterized by a component rich in mesophilous species of *Molinio-Arrhenatheretea* class and *Plantaginetalia majoris* order (*Mentha suaveolens* Ehrh., *Holcus lanatus* L., *Lotus tenuis* W. et K., *Trifolium repens* L., *Potentilla reptans* L.).

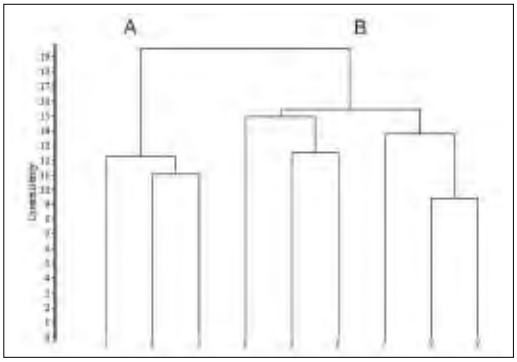

Fig. 23. Cluster analysis of fallow vegetation stands in the site "Serchio"

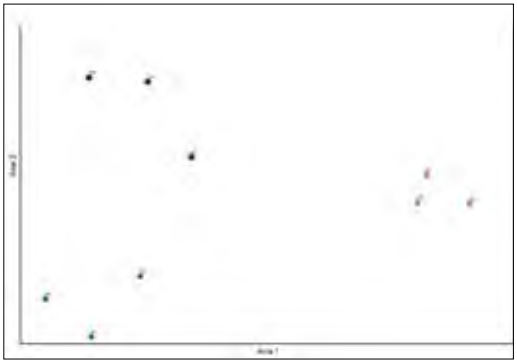

Fig. 24. Ordination diagram of fallow vegetation stands in the site "Serchio": in black and in blue, the stands characterized by a component rich in mesophilous (Molinio-Arrhenatheretea class) and hygrophilous (Phragmito-Magnocaricetea class). In orange, stands characterized by ruderal and nitrophilous perennial forbs and (Artemisietea vulgaris class). More explanation in the text.

Class and order include mesophile to wet, perennial, often manured meadows and pastures communities on deep and moist, periodically inundated soils (and, in this case, rich in organic nutrients). These stands are also characterized by the presence and relative abundance of *Phragmites australis, Mentha aquatica* L., *Lythrum salicaria* L., hygrophilous species of swampy and lacustrine environments (*Phragmito-Magnocaricetea*; Fig. 25, 26 and 27) and a sub-nitrophilous component of *Galio-Urticetea* Passarge ex Kopecký 1969 class [e.g. *Dipsacus fullonum* L. (Fig. 28), *Eupatorium cannabinum* L.]. The group on the right of the graph (corresponding to the cluster A) is formed by those stands characterized by a rich component of perennial and tall biennial forbs and grasses, ruderal and nitrophilous species growing on soils rich in organic matter (*Artemisietea vulgaris* Lohmeyer, Preising & Tüxen ex von Rochow 1951 class), with a clear dominance of *Artemisia verlotiorum* Lamotte (Fig. 29 and 30) (*Artemisietalia vulgaris* Lohmeyer in Tüxen 1947) order. A contingent of *Molinio-Arrhenatheretea* species is still present, indi-

cating a marked soil moisture, also during the summer period.
In Fig. 31 a scheme of the main plant communities occurring in this site has been provided.

Fig. 25. Lythrum salicaria

Fig. 26. Lythrum salicaria and Phragmites australis comm.

Fig. 27. Mentha aquatica

Fig. 28. Dipsacus fullonum

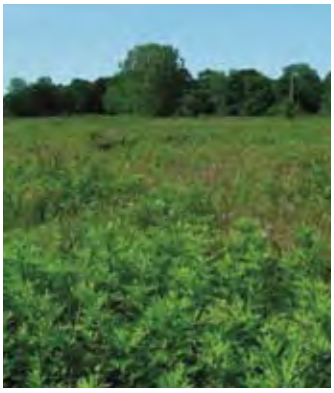

Fig. 29. "Serchio" site, fallow lands with Artemisia verlotiorum

Fig. 30. Artemisia verlotiorum

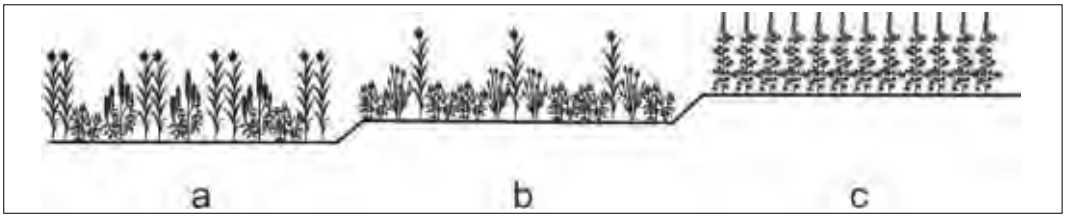

Fig. 31. An ideal schematic transect of the vegetation, from the low parts (left), subject to flooding periods, to the marginal areas (right). a) hygrophilous communities with Phragmites australis and Lythrum salicaria (Phragmito-Magnocaricetea class); b) meso-igrophilous communities with Mentha aquatica (Molinio-Arrhenatheretea class); c) nitrophilous communities with Artemisia verlotiorum (Artemisietea vulgaris class).

"Culatta" fallows

Fig. 32. "Culatta site", Fallow land

The plot selected in the "Culatta" site is a typical fallow land in an agricultural context (Fig. 32). According to the classification proposed by Rivas-Martinez et al. (2002), most of the species occurring in this site belong to the *Stellarietea mediae* Tuxen, Lohmeyer & Preising ex von Rochow, which comprises the annual grasses communities composed of ruderal, nitrophilous and semi-nitrophilous species (*Sinapis arvensis* L., *Papaver rhoeas* L., *Sonchus asper* (L.) Hill., *Phalaris paradoxa* L., *Alopecurus myosuroides* Hudson, Phalaris minor Retz., *Avena fatua* L., etc.). In this specific case, most of the species are typical of cultivated field weed communities. It is worth noting that in the late summer, when large part of the spring flowering species are withered, a group of annual species with late summer-autumn cycle appears. The most frequent are *Echinochloa crusgalli* (L.) P. Beauv. (Fig. 33), *Setaria viridis* (L.) P. Beauv. (Fig. 34), *Amaranthus graecizans* L.

In the analyzed plot, and also in the marginal areas surrounding fallow lands and cultivated fields of the "Culatta" site, a significant contingent of perennial, sub-nitrophilous and mesophilous species of the *Artemisietea vulgaris* Lohmeyer, Preising & Tuxen ex von Rochow class has been recorded [*Helmintotheca echioides* (L.) Holub, *Daucus carota* L., *Picris hieracioides* L. (Fig. 35), *Elymus repens* (L.) Gould subsp. *repens, Cirsium vulgare* (Savi) Ten., *Symphyotrichum squamatum* (Spreng.) G. L. Nesom, etc.].

Along the edges of the cultivated and fallow fields, in correspondence of the boundaries of drain ditches or of the hedges bordering the woody vegetation of the neighboring mixed

Fig. 33. Echinochloa crusgalli

Fig. 34. Setaria viridis

Fig. 35. Picris hieracioides

wood (Fig. 37), the vegetation is characterized by perennial, mesophilous herbaceous species, such as *Holcus lanatus* L. (Fig. 36), *Plantago major* L., *Rumex conglomeratus* Murray, *Verbena officinalis* L., *Sporobolus indicus* (L.) R. Br., *Cynodon dactylon* (L.)Pers., referring to the *Molinio-Arrhenatheretea* class and to the *Plantaginetalia majoris* order.

Fig. 36. Holcus lanatus

Fig. 37. "Culatta site", edges between cultivated fields and woody vegetation.

Mixed deciduous forest

As regards the forest vegetation, the mixed deciduous forest (Fig. 38) can be referred to the association *Fraxino angustifoliae-Quercetum roboris* Gellini, Pedrotti & Venanzoni 1986. Guide species is *Quercus robur* L. The herbaceous layer is characterized by the presence of *Iris fetidissima* L. (Fig. 39), guide species of the association. In the plot BM6, a variant in *Populus alba* L., as described by Tomei et al. (2004), has been found.

Fig. 38. The mixed deciduous wood

Fig. 39. Iris foetidissima

The analysis of the herbaceous layer allowed to highlight some ecological differences in the eight analyzed plots, otherwise not well detectable on the basis of the composition of the tree layer only. These differences are related to variations in flooding period and soil moisture. The most frequent and prevalent (in coverage) species are *Carex remota* L. and *Brachypodium sylvaticum* (Huds.) Beauv. Nevertheless, their distribution is very uneven when comparing different plots. *Carex remota* tends to prevail in those plots subject to

Fig. 40. Carex remota

prolonged flooding periods (BM3 and BM8), with coverage values of about 40-75% (depending on the total coverage of the herbaceous layer). In these cases, *Brachypodium sylvaticum* has not been recorded, or sporadically with low coverage rates (BM8), whilst a group of hygrophilous species, usually characteristic of wet meadoms or wetlands, such as *Juncus effusus* L., *Poa palustris* L., *Myosotis scorpioides* L., *Deschampsia cespitosa* (L.) P. Beauv., are present with high cover values. Specific attention has been applied to the analysis of the plot BM8. This plot is characterized by a central, bottom part subject to intense and prolonged flooding, and a surrounding part enduring a lower flooding period. The central part has a poor herbaceous layer, with large areas of bare soil and sparse clumps of *Carex remota* and *Juncus effusus* (Fig. 40 and 41). As the soil level raises, the grass coverage becomes higher, with *Carex remota* and other species such as *Carex sylvatica* L., *Rumex sanguineus* L., *Poa palustris* L., etc.

Fig. 41. Juncus effusus

In the most elevated stands with very short (or not) flooding and drained soils (BM4, BM5, BM6) *Brachypodium sylvaticum* coverage reaches high values (70-90%), becoming physiognomically prevalent. The trend of the ecological gradient linked to the soil moisture (and flooding period) and closely related to the composition of the herbaceous layer is schematically represented in Fig. 42, that is an ideal transect of the herbaceous layer, from the low parts, subject to very prolonged flooding periods, to the more elevated stands, subject to progressively less flooding and with well drained soils. The described hygrophilous mixed woods can been referred to the habitat 91F0 – "Riparian mixed forests of *Quercus robur*, *Ulmus laevis* and *Ulmus minor*, *Fraxinus*

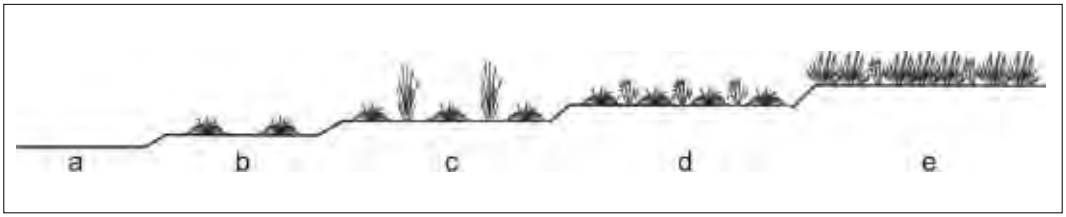

Fig. 42. An ideal transect of the herbaceous layer, from the low parts (left), subject to very prolonged flooding periods, to the more elevated stands (right). a) bare soil, long flooded in winter and wet in summer; b) sparse clumps of Carex remota; c) sparse clumps of Carex remota and Juncus effusus; d) high coverage of Carex remota and other species such as Carex sylvatica; e) dense herbaceous layer with Brachypodium sylvaticum.

excelsior or *Fraxinus angustifolia*, along the great rivers (*Ulmenion minoris*)", according to the Annex I of the Habitat Directive.

Forest with natural poplar

The forest with natural poplar is characterized by marked humid conditions, as evidenced by the high percentage of species belonging to the *Cyperaceae* family (17.3%). According to the vegetation analysis, the natural forest with poplar has to be referred to the association *Carici remotae-Fraxinetum oxycarpae* Pedrotti (1970) 1992. It is a lowland forests and the tree component consists largely of *Fraxinus angustifolia* Vahl subsp. *oxycarpa* (Willd.) Franco & Rocha Afonso. In this specific case, in the plot P1, subject to prolonged periods of submersion, there is an important presence of *Alnus glutinosa* (L.) Gaertner. *Populus* sp. pl. has a significant presence in all the three plots. In the herbaceous layer, *Carex remota* L. is the most frequent and sometimes dominant species, as described by Pedrotti & Gafta (1996). The comparison of the herbaceous layers of the three analyzed plots shows important differences in terms of floristic composition. Dominant species are *Mentha aquatica* L. in plot P1 (Fig. 43), *Agrostis stolonifera* L. in plot P2, while in plot P3 the species with the highest coverage is *Brachypodium sylvaticum* (Huds.) Beauv. This sort of gradient is due to a difference in soil moisture and to the different length of the flooding period in winter, with the longest duration in P1 and the shortest in P3 (Fig. 44). The species composition of the herbaceous layer of the plot P1, together with the high coverage (over 50%) of *Alnus glutinosa*, suggests the attribution of this vegetation to the association *Alno glutinosae-Fraxinetum oxycarpae* (Br.-Bl. 1915) Tchou 1946 that, according to Arrigoni (1990), represent the most evolved and mature hygrophilous planitial vegetation.

Fig. 43. Wood with natural poplar, plot P1. Mentha aquatica has a very high cover in the herbaceous layer

The described hygrophilous woods can been referred to the habitat 91F0 – "Riparian mixed forests of *Quercus robur*, *Ulmus laevis* and *Ulmus minor*, *Fraxinus excelsior* or *Fra-*

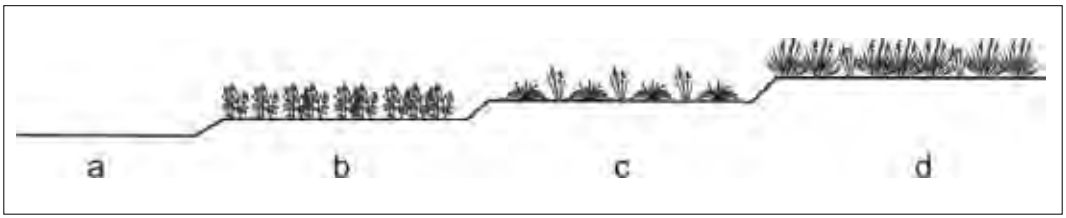

Fig. 44. An ideal transect of the herbaceous layer, from the low parts (left), subject to very prolonged flooding periods, to the more elevated stands (right). a) bare soil, long flooded in winter and wet in summer; b) herbaceous layer with Mentha aquatica; c) herbaceous layer with Agrostis stolonifera; d) dense herbaceous layer with Brachypodium sylvaticum.

xinus angustifolia, along the great rivers (*Ulmenion minoris*)", according to the Annex I of the Habitat Directive.

Pine wood

The pine wood located in the site "Colmate Bozzone", is an artificial forest, that is a *Pinus pinea* L. plantation which has replaced the primitive oak woods. Pine trees reach percentages of coverage of 70-80%. A well structure shrub layer has not developed. The presence of some shrubs, such as *Asparagus acutifolius* L. and *Smilax aspera* L., implies a potential development of a sclerophyllous shrub layer. The herbaceous layer consists of about 30 species. A group of mesophilous species, such as *Anthoxanthus odoratum* L., *Dactylis glomerata* L., *Elymus repens* (L.) Gould, *Gaudinia fragilis* (L.) P. Beauv., *Festuca arundinacea* Schreb. (*Molinio-Arrhenatheretea* class) comes from the neighboring environment of the wet meadows ("Lame"), while an amount of xerophilous and sub-nitrophilous species (*Bromus hordeaceus, Hypochoeris glabra, Vulpia bromoides, Urospermum dalechampii, Avena barbata, Brixa maxima*) indicates a general drought of this environment compared to the other two examined forest environments.

Fig. 45. Ordination diagram (NMS) of the herbaceous layer of the 1000 m² plots selected for the woody vegetation. In black, the mixed wood; in blue, the wood with natural poplar; in red the pine wood.

The ordination analysis applied to the herbaceous component of the 1000 m² plots selected for the woody vegetation, shows very clearly how the pine wood plot definitely segregates, on the right of the graph, from those of hygrophilous forests, on the left (Fig. 45).

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Chapter 3

Assessment of local biodiversity

3.2 - Macroinvertebrate diversity

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Introduction

To create a database of the macroinvertebrate community present in the Migliarino - San Rossore - Massaciuccoli Regional Park a literature review was undertaken. More than 205 papers were consulted and these mainly regarded entomological studies focusing on certain orders or families. This favoured the better knowledge of some groups compared to others but gave little information on the diversity levels of the single habitats. Through this consultation a list of species was created. For each species information such as taxonomy, author descriptor, the area of collection and literature reference was collected. As a result a total of 1456 species were described within the territory of the park of which the most studied taxa were the Arthropoda Hexapoda. These made up 91.7% of the total species. The Coleoptera was the most studied order with 632 species, followed by the Lepidoptera (308 species), the Hymenoptera (191 species), the Thysanoptera (59 species), and the Diptera (42 species). In this list of species, 8 species (Coleoptera: *Cerambyx cerdo*, *Lucanus cervus*, *Osmoderma eremita*; Lepidoptera: *Callimorpha quadripunctaria*, *Lycaena dispar*; Odonata: *Lindenia tetraphylla*; Gastropoda *Anisus vorticulus*, *Vertigo moulinsiana*) were included in the Council Directive 92/43/CEE of the European community 21/05/1992 for which their conservation requires the designation of special areas of conservation. Of these *Osmoderma eremite* and *Callimorpha quadripunctaria* were considered priority species. Furthermore, 6 species (Coleoptera: *Cerambyx cerdo*, *Osmoderma eremita*; Lepidoptera: *Lycaena dispar, Zerynthia polyxena*; Odonata: *Lindenia tetraphylla*; Gastropoda *Anisus vorticulus*) were considered of interest for the international community and were designated as species in need of strict protection (Council Directive 92/43/CEE of the European Community 21/05/1992). Within the list there were 43 species (5 Mollusca, 5 Lepidoptera, 26 Coleoptera, 5 Odonata, 1 Diptera, 1 Hymenoptera) considered of regional interest according to Regional Law 56/2000 for which their conservation requires the designation of sites of regional interest (SIR).
Within the DEMETRA Project the aim of the study was to evaluate species richness,

abundance, and macroinvertebrate diversity in the different study sites and, in particular, on the inflorescences of sunflower, maize, rapeseed crops and of wild poplar trees.
For this reason standard sampling methods replicable both in space and time were used. These methods were very efficient and adequate for ecological studies but were less effective for entomological inventories.
Another aim of the study was to investigate on the role of the experimental crops (possible GMOs) within the food webs.
The isotope signatures of carbon and nitrogen were determined for the macroinvertebrates associated to the inflorescences using stable isotope analysis, a technique commonly used in several fields of study such as anthropology (Boyd et al. 2008), archaeology (Gil et al. 2011), agriculture (Briones et al. 2001) ecology (Colombini et al. 2011) and for conservation purposes (Darimont et al. 2007).
This technique permits the determination of the trophic levels of the different species and to appoint species to different trophic guilds.

Materials and Methods

Study sites

The analysis of macroinvertebrate diversity was conducted in several sites within the three study areas of the Migliarino - San Rossore – Massaciuccoli Regional Park.

Area 1, Zone of the Massaciuccoli Lake

Lake system

The area in which the samplings were carried out consisted in a large wetland zone of the Massaciuccoli lake bordering the arable fields to the south.
Within this zone several habitats were considered (Fig. 1):

a) a reed community with dominant *Phragmites australis* that had been burnt (Fig. 2),
b) banks of the so-called “hunter’s open space”,
c) external bank of the Anghetto channel bordering the agricultural fields,
d) internal bank of the Anghetto channel with the threatened Silk Vine *Periploca graeca* (Fig. 3),
e) peat bog with dominant *Sphagnum* sp. moss

Two different points were sampled in each of the five study habitats.

Area 2, Serchio zone

Ontanelli Locality:

Incolto S21 (non cultivated field)
In this locality invertebrate samples were collected in a non cultivated field where the dominant plant species was *Artemisia coerulescens* (Fig. 4).
Previously this field had been occupied by a cultivated poplar tree variety. The study was carried out in the three 30 x 30 m plots in which the botanists had conducted their samplings.

Fortino Nuovo Locality

Woodland with wild poplar trees :

In this site (Fig. 5) sampling occurred in 2 different points for each of the 30 x 30 m plots analysed by the botanists.

Area 3, Arno zone

Culatta Locality

Plot A6

a) portion with sunflower crops (Fig. 6)
b) portion with maize crops (Fig. 7)
c) wild non cultivated portion
d) portion with rapeseed crops
e) portion with potato crops

In this plot a total of 5 sampling points were assessed at the centre of each type of crop.

Culatta Locality

Mixed broadleave forest

In this site (Fig. 8) sampling occurred in 4 points of the 30 x 30 m plots analysed by the botanists.

Colmate del Bozzone Locality

Pinewood

Sampling was carried out in 3 points within a fascia of pine trees of *Pinus pinea* occurring between the road running North-South and the wetland meadow (le Lame) reaching the sea.

Colmate del Bozzone - Lame Locality

Wet meadow

Habitat Natura 2000: 6420 (Mediterranean tall humid grasslands of the *Molinio-Holoschoenion*)

In this site (Fig.s 9 and 10) invertebrate species were collected in 7 points located next to the 10 x 10 m plots analysed by the botanists.

Sampling methods

To analyse the macroinvertebrate diversity of each study site a standard system of pitfall traps was used. Samples were collected in spring and autumn seasons. Each trap consisted of a plastic cup (9 cm in diameter) pushed to the ground surface and provided by four intercepting bands 10 cm in height which increased the interception capacity (Fig. 11). All pitfall traps with ethylene glycol were kept active 48 consecutive hours so as to capture both nocturnal and diurnal invertebrates.

During the spring/summer season to study the faunal diversity associated to the flowers and to examine how the population changed through time the inflorescences of sunflower, maize and rapeseed crops were collected during the blooming period. In each cultivated plot at noon 9 inflorescences were gather with the associated fauna. In the case of the sunflower crops the same number of inflorescences was collected immediately after sunrise and after sunset for a total of 27 inflorescences sampled. This method was applied so as to verify the possible differences in the exploitation of the sunflowers by the macroinvertebrates according the different moments of the day which corresponded to different sun intensities which were important especially for flying insects. The inflorescences with associated fauna were collected in plastic bags and then frozen for a few minutes to facilitate animal collection.

To examine how macroinvertebrate diversity changed in relation to the percentage of blooming of the field of the different crops weekly samplings were carried out so as to include the entire blooming period. At the end of the winter/ beginning of the summer season the same type of analysis was carried out for male and female inflorescences of poplar trees (*Populus canescens*). In this case the poplar trees that were investigated were wild poplars belonging to a forest at Fortino Nuovo Locality and to the banks of the Massaciuccoli Lake. All these trees had been previously identified as males or females and geographically located with GIS. All specimens captured with these systems were preserved in 75% alcohol and then sorted to species level. When it was not possible to identify the species individuals were sorted with the criteria of the morphologically recognisable taxonomic units (RTUs) (Kruger & McGavin, 1997). This method consisted in subdividing the specimens in orders or at family level and then in grouping the different species with conventional names (family name 1, 2, 3 etc.). RTUs will be called “species” throughout the text.

To analyse trophic webs macroinvertebrates were collected both with pitfall traps and with active researches on the inflorescences of the different crops (Fig. 12). In each site an adequate number of pitfall traps was set according to the type of habitat and keep operative for 24 hours. Captured individuals were then frozen, oven dried and preserved at a temperature of 37°C. For isotope analysis of $\delta\ ^{13}C$ and $\delta\ ^{15}N$ samples were prepared crushing with a mortar 5 individuals of each species to homogenise the sample. An aliquot of ca. 0.3 mg of that composite sample was used for isotopic determination.

Carbon and nitrogen isotopic signatures were measured from the gasses evolved from sample combustion in a Finnigan Delta S isotope ratio mass spectrometer (Conflo II interface). Isotopic values are reported in the δ notation relative to the standards Vienna Pee Dee Belemnite for carbon
and air for nitrogen ($\delta sample = [(R_{sample}/R_{standard}) - 1] \times 1000$, $R = {}^{13}C/{}^{12}C$, or $R = {}^{15}N/{}^{14}N$).

Statistical analysis

All collected data were included in an elettronic database (Microsoft Excel) and then

analysed statistically (Colombini et al. 2002). Diversity indices of macroinvertebrates were calculated in the two seasons for the different localities and for the inflorescences of the experimental crops and poplar trees . The α diversity index of Fisher et al (1943) was used. To analyse the evenness of the community, Pielou's (1978) evenness index through Brillouin (1962) index was used. To express the abundance of the commonest species as a fraction of the total number of individuals, Simpson's (1949) dominance index was calculated. The statistical package Open Source BioDiversity PRO was used to calculate diversity indices.
For qualitative comparisons of macroinvertebrates of the different localities in the different seasons and among inflorescences of different crops, Renkonen's (1938) Similarity Index was computed. This was calculated as the percentage of species in common over the total number of species.

Results

A total of 443 species were collected in the different study sites on a total of 11782 specimens captured with the standard system of traps (pitfall traps, captures on the inflorescences of sunflower, maize, rapeseed crops and of wild poplar trees). In this case the most represented taxa were the Arthropoda Hexapoda with 77.6 % of species of which the Coleoptera order had the highest species richness (n=145). However in the study areas also the Hymenoptera and Diptera orders were important in species number presenting a total of 64 and 42 species respectively.

A) Sampling with pitfall traps

Comparing samples obtained in the different study sites (Table 1) the highest capture numbers were mainly recorded during the period of spring and the wet meadow (Le Lame) of the Colmate del Bozzone showed the highest value. Contrarily the lowest captures occurred in the experimental plots of sunflower and maize. Also considering species number the highest values were recorded during spring season with a total of 66 species at the Incolto S21 (non-cultivated field) site at the Ontanelli locality, followed by the lake system at the Massaciuccoli Lake with 58 species. In autumn a decrease in both abundance and species richness was generally found in all study sites except for the site with the experimental crops where the opposite occurred. In this season the highest value for species richness was registered for the wetland area of Le Lame.
In the study sites the mean δ diversity values were 13.95 and 11.41 respectively in spring and in autumn. When the sites were considered individually in some cases δ diversity indices was higher in spring when compared to autumn, in other cases the opposite occurred (Table 1). The highest δ diversity (31.75) was found in spring at the Incolto S21 (non-cultivated field) site of the Ontanelli locality, followed by the lake system of the Massaciuccoli Lake (19,84). Whereas the lowest δ diversity value occurred in the Culatta

Locality Plot A6 in the potato field (4.93) in spring.
Generally speaking the values of the Pielou index (Table 1) registered during spring were higher than those obtained during the autumn season. Instead the values of Simpson's index showed the opposite tendency. In particular in spring the wet meadow of Le Lame showed the lowest Pielou value (0.424) and the highest dominance index of Simpson (0.454) indicating the presence of a macroinvertebrate community less uniform due to the occurrence of some dominant species. The opposite occurred in spring in the field where sunflower crops were cultivated. In this case the macroinvertebrate community was evenly distributed as demonstrated by the high Pielou index and no dominant species occurred (see low value of Simpson index).
When the macroinvertebrate community of the two wetland areas were compared, at the Lake system of the Massaciuccoli Lake a species belonging to the Amphipoda was found to be dominant whereas at the wet meadow (Le Lame) of the Colmate del Bozzone two different species of the Araneae were most abundant according to the season analysed.
In the two non cultivated sites that were studied the Formicidae family was dominant in spring both at site of the Ontanelli Locality and of that of the Culatta Locality, whereas in autumn the Isopoda were most important at the first site and the Heteroptera at the second one.
When the different types of forests were compared the species belonging to the Araneae and the Formicidae were the most important macroinvetertebrates. In spring both the forest with wild poplar trees at the Fortino Nuovo Locality and the mixed broadleave forest at the Culatta Locality showed dominant Araneae species followed by the Formicidae species. In autumn the latter became the most important together with species belonging to the Collembola order. To be noted that the Araneae and the Formicidae were the most abundant species also for the stone pine forest at the Colmate del Bozzone even if this locality was sampled only during the summer season.
In spring the Orthoptera order was the most abundant at the Culatta Locality where the experimental plots of sunflower, maize and potato crops were set. In autumn even if the orthopterans were still important there was an increase of coleopterans belonging to the Carabidae family. The latter together with a collembolan species were the most abundant macroinvertebrates collected within the rapeseed crops during spring.
On a total of 269 species captured with pitfall traps in all sampled sites a certain number was unique for a single site and for a certain season (Table 2). For example at the lake system of the Massaciuccoli Lake in autumn 24 species over a total of 58 species were unique for this locality and for this season whereas in spring only 2 species were unique over a total of 29 collected species. In autumn 50 % of the unique species was composed by Coleoptera Carabidae and by Araneae. In spring no unique species occurred in the experimental crops of sunflower and maize.
Comparing the percentage of similarity of the different species in the different study sites calculated with Renkonen's Similarity Index a percentage of similaritity over 20 % was obtained for 17 comparisons (Table 3). It should be noted that the highest similarities were

registered within similar environments as was the case of the fields with the experimental crops (Table 3: cases 7-14) or in the semi-natural environments (Table 3: cases 15-25). The highest species overlap was recorded in spring at the Culatta Locality between the fields of sunflower and maize crops (53.3%). As a matter of fact higher similarity values were obtained when faunal communities of the two cultivations were compared within the same season rather than when comparisons were made between communities of the same crop in the two different seasons. In the case of semi-natural environments high similarities in the faunal communities were obtained between the forest with wild poplar trees at the Fortino Nuovo Locality and the mixed broadleave forest at the Culatta Locality in the two seasons. Instead a very small overlap occurred when the macroinvertebrate communities of the wet meadow of Le Lame were compared in the two seasons.

B) Sampling on the inflorescences of poplar, sunflower, maize and rapeseed crops

Analysing the macroinvertebrate samples collected on the inflorescences (Table 4) the highest abundance and species richness were registered for the fauna associated to the sunflower crops (respectively n=4397 and n=109) whereas the lowest values were obtained for the inflorescences of male poplar trees of the Massaciuccoli Lake (respectively n=58 e n=13)(Fig. 13 and 14). The inflorescences of male poplar trees also showed the lowest δ diversity values especially at the Fortino Nuovo Locality (δ=3.42). Furthermore, the δ diversity value obtained for the inflorescences of female poplars at the Massaciuccoli Lake was higher than that of males (respectively δ=9.36 and δ=5.20) and the macroinvertebrate community was quite uniform (see high Pielou index). The higher value of Simpson dominance index recorded for male poplar trees indicate the presence of a dominant species. As for the inflorescences of the three crops in the Culatta Locality the highest δ diversity value was registered for the sunflower crops, which also showed a fairly uneven community (see Pielou index), In this case the value of the dominance index suggests the presence of dominant species.

In the lake system at the Massaciuccoli Lake sampling on the inflorescences of male and female poplar trees was carried out for four consecutive weeks during the blooming season. Over 58 captured individuals a total of 13 species were collected on male trees, whereas on female trees species richness was higher with 26 species present over a total of 141 collected individuals. Also the composition of the macroinvertebrate community differed between male and female trees with Thysanoptera and Araneae (respectively 48.3 and 32.7% of total captures) mainly on inflorescence of male trees and Chrysomelidae, *Altica oleracea* (12%) a dipteran (11%) and a coleopteran species (*Neocrepidodera brevicollis*)(9,7%) mainly on inflorescence of female trees.

At the Fortino Nuovo Locality of the Serchio zone samplings were carried out for three consecutive weeks only on the inflorescences of male poplar trees during the blooming season. On a total of 363 captured individuals 16 species occurred (Table 4). A high

abundance of larvae of Diptera Tabanidae were collected on male poplar trees reaching 75 % of total captures. These were followed by a species belonging to the coleopteran Coccinellidae family with 9.4 % of captures.
In the Culatta Locality of the Arno zone samplings were carried out on the inflorescences of the sunflower crops for five consecutive weeks during the blooming season for a total of 135 samples. On a total of 4397 captured individuals 109 species occurred (Table 4). The heteropteran *Scoloposthetus decoratus* was the most abundant species (39.9 %) followed by the coleopteran Latridiidae *Cortinicaria gibbosa*, the homopteran *Macrosteles variatus* and by the heteropteran *Cymus melanocephalus*, all with similar capture numbers (respectively 14.2, 13.8 e 13.3% of total captures).
Samplings on the female inflorescences of maize crops were carried out for four consecutive weeks for a total of 36 samples. On a total of 908 captured individuals 63 species were found (Table 4).
The larva of the Lepidoptera, *Ostrinia nubilalis*, a target species in the case GMO maize plants, was the most abundant sampled species (22.7% of total captures). This was followed by the coleopteran Latridiidae *Cortinicaria gibbosa*, the heteropteran *Scoloposthetus decoratus* and by a species belonging to the Thysanoptera order (respectively 20.7, 18.1 and 16 % of total captures).
Samplings on the inflorescences of rapeseed crops were carried out for four consecutive weeks for a total of 51 samples. On a total of 2022 captured individuals 56 species were found (Table 4). A species belonging to the Thysanoptera order was the most abundant (36.1 %) followed by the adults and larvae of the coleopteran Nitidulidae *Meligethes aeneus* and by the curculionid *Ceuthorrhyncus napi* (respectively 21.9, 12.5 and 7.5% of total captures).
A total of 16 unique species were collected on the inflorescences of poplar trees of which 2 were unique species for the male and 9 for the female trees. Twenty-two unique species were found for rapeseed crops whereas for sunflower and maize crops the unique species that occurred were respectively 44 and 19. There were also 22 unique species that were in common between the latter two cultivars. When considering abundance and species richness 80.62 % of the captured individuals and 33.94 % of the species of the sunflower inflorescences were in common respectively with the captured individuals and the species of the maize crops.
On the other hand 95.48 % of the captured individuals and 58.73 % of the species of the maize inflorescences were in common respectively with the captured individuals and the species of the sunflower crops.
Comparing the percentage of similarity of the different species collected on the inflorescences calculated with Renkonen's Similarity Index (Table 3: 1-6) only for 2 comparisons (maize versus sunflower and female poplars versus male poplars at the Massaciuccoli Lake) the percentage of similaritity was over 20 %.
The small similarity index registered for male trees of the Massaciuccoli Lake and those of Fortino Nuovo should be noted.

C) Temporal variation of macroinvertebrate diversity on the sunflower inflorescences

Samplings on the inflorescences of the sunflower crops were conducted every 7 days and each time 9 inflorescences were collected at three different hours of the day (06.00, 12.00 e 18.00). This experimental design was carried out to see if there were differences in the macroinvertebrate community of the inflorescences according to the period of blooming of the experimental crops and to the time of the day. The results of the study indicate that the Heteroptera order was the most abundant followed by the Homoptera and Coleoptera (Table 5 A). The Heteroptera reached a peak of abundance during the fifth week with a value two or three times higher compared to those of the previous weeks. A similar trend appeared for the Coleoptera that peaked during the fifth week but with a value ten times higher the previous ones. Instead for the Homoptera, even if the total occurrence was comparable to that of the Coleoptera (respectively 15.83 and 15.42 %), their presence was never higher than 6 % (second week) and decreased to 1.2 % during the last week of sampling. Even when considering the hourly variations (sunrise, noon and sunset) of the macroinvertebrate community on the inflorescences a set of different behaviours occurred according to the different orders (Table 5 B). The Heteroptera together with the Araneae and other orders were equally present on the inflorescences at the different hours of the day whereas the Homoptera and Coleoptera mainly occurred at sunrise. In the case of the Thysanoptera the highest occurrence on the sunflower's inflorescences was found at midday.

D) Analysis of trophic webs

The analysis of stable isotopes of $\delta^{13}C$ e del $\delta^{15}N$ carried out to study food webs showed that sunflowers ($\delta^{13}C$= -26.94 ‰), rapeseeds ($\delta^{13}C$= -27.12 ‰) and poplars ($\delta^{13}C$= -28.21 ‰) all belonged to the C3 plants (Colombini et al 2011, Jahren & Kraft 2008) whereas maize ($\delta^{13}C$= -12.38 ‰ and $\delta^{13}C$= -12.36 ‰ respectively for shoots and roots) to the C4 plants (Fig. 15). On the whole, considering all data together (Fig. 15), it was possible to point out two trophic chains (a, b) originating from different trophic levels. In the case of the trophic chain "a" the lowest values of $\delta^{15}N$ were found for primary consumers such as Polmonata molluscs (0.46‰), collembolans (-1.9 ‰) and blattids (-1.1 ‰) that feed on fungus, terrestrial unicellular algae, bacteria etc. In the case of the trophic chain "b" the lowest values of $\delta^{15}N$ again occurred for primary consumers such as lepidopteran Noctuidae (1.6 ‰; 2.3 ‰; 3.1 ‰) but these directly fed on C3 plants. In both chains the highest values of $\delta^{15}N$ belonged to macroinvertebrate predators of the Araneae and Coleoptera orders. It should be noticed that only one Lepidoptera species (larvae of *Ostrinia nubilalis*) was found associated to the C4 plants.
Lets now consider the two trophic chains starting from the orders that have species which

are primary consumers. Of the Orthoptera order (Fig. 15) three species belonging to three different families (Acrididae, Tettigonidae and Grillydae with increasing $\delta^{15}N$ values) were investigated. The species of the Grillydae family showed values for $\delta^{15}N$ (5.95 ‰) and $\delta^{13}C$ (-27.4 ‰) slightly higher of the hypothesized trophic enrichment for poplars (see box)[1]. The species of the Tettigonidae family ($\delta^{15}N$ =4.81 ‰) was more in the area of those macroinvertebrates associated to rapeseed crops whereas the species of the Acrididae family didn't seem to be associated to any of the food chains starting from the three experimental crops.

As regards to the Lepidoptera order, all primary consumers, species belonging to the Noctuidae, the Pieridae and the Piralidae families (Fig. 15) were considered. The species of the first two families all occurred in areas along the food chain of the C3 crops with $\delta^{15}N$ values ranging from 1.6 ‰ to 5.1 ‰ and $\delta^{13}C$ values from 25.9 ‰ to -28.5 ‰. *Ostrinia nubilalis*, a species of the Piralidae family, had $\delta^{15}N$ (3.29 ‰) and $\delta^{13}C$ (-11.5 ‰) values that clearly indicated it total dependence on maize as a food source.

The Hymenoptera order included species which were both primary and secondary consumers. Four different species were analysed. Of these two belonged to the Formicidae, one to the Apidae and another one to the Vespidae family (Fig. 15). The species belonging to the first two families had $\delta^{15}N$ values ranging from 0.75 ‰ to 3.9 ‰ and showed that they were primary consumers but didn't belong to the food chain starting from the C3 plants ("b"). Instead, the species of the Vespidae family had a definitively higher $\delta^{15}N$ value (7.3 ‰), showed a trophic level typical of a secondary consumer and was associated to the food chain "b".

The Diptera order included species which were both primary and secondary consumers. Six different species belonging to the Chironomidae, Culicidae, Simuliidae, Bibionidae, Syphidae and Tabanidae families were analysed (Fig. 15). Even if the tabanid was a predator species it had a $\delta^{15}N$ value (4,8‰) lower than those of the Chironomidae (6.4 ‰) and Simuliidae (7.7 ‰) species which were both primary consumers. Remarkable was the trophic enrichment obtained for the Simuliidae species which was exactly 1‰ for $\delta^{13}C$ and 3.4 ‰ for $\delta^{15}N$ with respects to the sunflower crops showing a direct correlation between the food source and its consumer.

From a trophic point of view the Coleoptera order (Fig. 16) is extremely heterogeneous as it includes species which are primary consumers, detritivores with wide food ranges and secondary consumers with high trophic levels. The isotope signatures obtained for the different analysed species had $\delta^{15}N$ ranging from -1.2 ‰ (Cerambicidae) to 11.6‰ (Carabidae) which could be found along the two food chains (a, b). In fact a group of 4 species belonging to the Cerambicidae, Buprestidae, Tenebrionidae and Oedemeridae families were primary consumers with very low isotope signatures (from -1.2 ‰ to1.03 ‰) and occurred along the food chain "a". Another group of 12 species, all secondary

[1] In a wide range of consumer-food pairs, the isotopic content of an animal has been found on average to be 1‰ (δ13C) and of 3,4‰ (δ15N) higher than that of its food (DeNiro and Epstein 1978,1981). So the boxes of Fig.s 15-16 indicate the trophic enrichment between food sources (sunflower, maize, rapeseed and poplar) and possible primary consumer.

consumers and belonging to both food chains had isotope signatures from 4.2 ‰ (Carabidae) to 11.6 ‰ (Carabidae). However it should be stressed that species which are members of a same family often have different isotope signatures which sets them at different trophic levels. Furthermore, there were four predator species of the Carabidae, Coccinellidae and Cicindelidae families which had $\delta^{15}N$ values between 4.2 ‰ and 6.9 ‰ and $\delta^{13}C$ values between -26.4 ‰ and -24.2 ‰. These species were placed along the food chain starting from the C3 plants ("b") and in particular from the rapeseed and sunflower crops. Interesting was also the species of the Silphidae family which was placed along the food chain close to the previous group. This species was not a predator but a necrophagous species feeding on vertebrates. In this case the $\delta^{15}N$ value (8.3 ‰) was lower than that of two species (a Staphylinidae 9.8 ‰ and a Carabidae 11.6 ‰) which preyed on other macroinvertebrates.
Also for the Araneae order two groups of predator species clearly fitted in well along the two trophic chains (Fig. 15). The first group of five species belonging to the Linyphiidae, Thomisidae, Agelenidae, Cybaeidae and Lycosidae families had isotope signatures of $\delta^{15}N$ ranging from 2.17 ‰ to 4.41 ‰ and of $\delta^{13}C$ from -26.14 ‰ to -22.6 ‰ and were placed along the food chain "a". Instead, the second group was placed along the trophic chain "b" to which the experimental crops were linked. This group was composed by four species with isotope signatures of $\delta^{15}N$ ranging from 3.7 ‰ to 7.4 ‰.
Finally a heterogeneous category called "others" grouped species belonging to different orders and different trophic levels (Fig. 15). Also in this case it was possible to associate the different species to the two food chains. A group of species was placed along the food chain "a" of which the predator species of the Chilopoda order showed the highest $\delta^{15}N$ value. Instead along the food chain "b" two primary consumers of the Dermaptera and Homoptera orders had lower levels compared to the two predator species of the Anisoptera and Zygoptera orders. It should be pointed out that the Homoptera species can be linked to the rapeseed food web and this species can be preyed on by the Zygoptera species.
Some data regarding isotope signatures of passerine birds were collected from the literature (Evans et al. 2012). The isotope signatures of birds that feed in flight (House Martin and Common Swift) and on the vegetation (Willow warbler and Wood warbler) were compared to those of macroinvertebrate predators such as spiders and coleopterans. The results indicate that trophic levels were quite similar and comparable.

Discussion and Conclusions

In spring the general tendency of a high abundance and species richness of macroinvertebrates was mainly due to the life cycles of the single species but also to the consistent number of plants in blossom especially in the open areas, such as those of the wet meadows of Le Lame and the non-cultivated areas, more than those of forest areas. On the contrary the experimental plots of sunflower and maize showed higher abundance and species richness in autumn. As a matter of fact in spring due to the ploughing of the

fields to prepare for the crop cultivation and to the presence of monocultures the diversity of habitats was extremely reduced. In autumn, instead, the area was subjected to an increase in its complexity due to the presence of the crops that were left *in situ* and to the growth of wild herbaceous plants and/or weeds

The highest α diversities were registered during the blooming period in homogeneous environments with a high vegetation cover and complexity (like meadows and non-cultivated areas) but also in heterogeneous areas with ecotonal characteristics such as those along the banks of the Massaciuccoli Lake. In the latter case the drastic decrease recorded for the α diversity value in autumn was related to the lower water level of the lake recorded in this period and to the consequent drying up of the different habitats. In the case of the experimental potato field the low α diversity value registered in spring was due to the small size of the cultivated plot and to the total absence of a vegetation cover of the surrounding areas that had been ploughed.

Generally speaking macroinvertebrate communities were different according to the study sites, however differences occurred also between similar environments such as wetlands, non-cultivated areas, forests and cultivated plots.

The differences found in the macroinvertebrate communities of the two wetlands (Massaciuccoli Lake and wet meadow Le Lame of the Colmate del Bozzone) can be related to a number of factors such as the environmental heterogeneity due to different hydrological regimes, the presence of certain plant species with higher or lower vegetation cover and the type of management. In fact Le Lame is a homogeneous environment covered by tall humid grasslands (*Molinio-Holoschoenion*) characterised by the presence of water (due to rainfall) during the winter and beginning of the spring season. When the wetland starts to dry up Park authorities mechanically mow the area to maintain adequate characteristics for certain bird species. Instead the environment of the Massaciuccoli Lake was definitively more heterogeneous and different habitat typologies, such as the wetland zone with *Phragmites australis*, the ecotone between the common reed and the lake's waters, the two banks of the lake (one with grass and the other with *Periploca graeca*) and the peat bog with *Sphagnum* sp., were investigated. Each typology was subjected to a different type of management. For example the wetland area with common reed had been burnt the previous autumn season, the vegetation of the "hunter's open space" was regularly cut each year and the lake's banks with grass was mown by the local authorities in charge of water management. Semi-natural conditions occurred only in the case of the banks with *Periploca graeca* and of the peat bog even though there were variations due to the water levels of the lake.

The differences found in the macroinvertebrate communities of the two non-cultivated sites, one at Ontanelli and the other at the Culatta Locality, were mainly related to the different land uses that occurred previous the abandonment. In fact at the first locality the plot had been used for a poplar stand whereas at the second for cereal cultures. The differences in land use brought to differences in the plant species that successively colonised the area and consequently to the associated macroinvertebrate communities.

At the Ontanelli Locality *Artemisia coerulescens*, a plant with shrub characteristics and typical of degraded clay and salty soils, became the dominant species when sampling occurred, whereas at the Culatta Locality the area developed a very diverse herbaceous vegetation cover. In this case, contrarily to what could be expected, the higher diversity of macroinvertebrates was found where the vegetation was more homogeneous.
As regards to the different types of forest that were studied, woodland with wild poplar trees at Fortini Nuovo and mixed broadleave forest at the Culatta Locality, especially in spring small differences in the macroinvertebrate communities were found. These two forest had common traits and in particular both had large areas flooded during the winter, both had a limited shrub understorey and both were characterised by large quantities of dead wood in relation to the type of management that left dead trees and branches *in situ*. Also for the stone pinewood the differences in the macroinvertebrate community were quite small even if samplings had been carried out during the summer. These were probably related to the similar type of management that was operated in the area.
The lower diversity values found in spring for the experimental crop fields compared to all other study sites were due to the preparation of the fields before sowing, the mechanical weeding and to the presence of monocultures. In fact the first two operations disturbed and homogenised the superficial layers of the soil, eliminating the herbaceous vegetation and had devastating consequences on the macroinvertebrate communities. The absence of unique species in the fields with crops was related to the type of land use which implicated each time the colonisation of the microinvertebrates from the surrounding areas. On the contrary the great number of unique species found for the Massaciuccoli Lake was related to its peculiar environmental characteristics and to its geographical distance from all the other compared sites. Naturally macroinvertebrate diversity was more similar when comparisons were made between corresponding sites within the same season rather than when comparing the same site in two different seasons: e.g. woodland with wild poplar trees at Fortini Nuovo and mixed broadleave forest at the Culatta Locality in spring; woodland with wild poplar trees at Fortini Nuovo in the two seasons.
To evaluate possible hybridization of wild plant species with GM crops of sunflower, maize, rapeseed and poplar through insects as possible vectors, the macroinvertebrate community associated to the inflorescences of crops was studied through time. This was done in order to understand how the communities changed according to the blooming period. The study showed that there was an elevated number of macroinvertebrates on the most striking inflorescences, like sunflowers and rapeseeds (dependent on insect pollination) for which there was also a high number of species. Of all the species occurring on the experimental crops, of which many were fliers, a great number of unique species was found. Of these a consistent number was unique for both sunflower and maize crops confirming the high similarity obtained between the two communities when the total species numbers were considered (see Renkonen Similarity Index). Instead species richness and macroinvertebrate diversity on poplars, which depended on wind pollination, always had lower values compared to plant species with insect pollination. For poplars

the major differences occurred between male and female trees of the same locality rather than between plants of the same sex but of different localities. Furthermore, a low number of unique species was found associated to trees of both sexes which perhaps indicated a random presence on the inflorescences.
The temporal analysis carried out for the sunflower's inflorescences showed that there were changes in the composition of the macroinvertebrate community both at a large temporal scale as the blossoming of the crops evolved through time and on a smaller scale throughout the different hours of the day. In the first case this can depend on the higher or lower attractiveness of the entire field which varies according to the general state of the crop's growth. The hourly variations of the macroinvertebrate community instead indicate different strategies in the use of the sunflower's inflorescence. The constant presence on the inflorescence can indicate the total dependence of a species to this microhabitat whereas a presence in the early morning hours or in the central part of the day shows that it is not a priority habitat. The occurrence of certain species in the first hours of the day shows that the inflorescences can be exploited as a nocturnal refuge and that foraging occurs elsewhere. In other cases the sunflower's inflorescences are used as a food source but not as a nocturnal resting place as was the case of bee species. These hypothesis were confirmed by the elevated number of species and individuals that were found associated to the sunflowers.
The study on the food webs associated to the experimental crops showed the existence of two food chains originating from two different primary sources. A primary food source presumably was composed by fungus, unicellular algae, mosses, lichens and bacteria (Colombini et al 2011) whereas
another one was based on C3 plants including sunflowers, rapeseeds and poplars. Another primary food source was composed by the C4 plants to which the maize crop belonged. In the two trophic chains primary consumers can be selective towards a specific food source but at higher levels consumers become less selective and there is the possibility of an exchange between the two chains. Only the larvae of 5 the lepidopteran *Ostrinia nubilalis* was found as a primary consumer of maize but its direct predator species was not detected. Predators such as spiders and coleopteran carabidae occurred at the top of the two food chains and showed trophic levels similar to those of passerine insectivores feeding while in flight or directly on the insects of the inflorescences. Since bird species have greater feeding ranges compared to insect predators they can forage at geographically distant areas within similar food chains. This means that insectivore birds feeding directly on the inflorescences become possible pollen vectors operating at higher distances compared to insect pollinators.

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Table 1. Diversity indices calculated for the two seasons at the different sites (n= numbers; sp = species).

	Total n	sp n	Alpha	Shannon H'	Shannon H max	Pielou J'	Simpsons D
MASSACIUCCOLI							
Lake spring	349	58	19.041	1.319	1.763	0.748	0.078
Lake autumn	248	29	8.52	1.012	1.462	0.692	0.167
ONTANELLI							
Non cultivated S21 spring	222	66	31.75	1.553	1.82	0.854	0.048
Non cultivated S21 autumn	160	33	12.61	1.106	1.519	0.728	0.176
FORTINO NUOVO							
Woodland with poplars spring	343	46	14.295	1.193	1.663	0.717	0.102
Woodland with poplars autumn	182	27	8.769	0.751	1.431	0.525	0.39
CULATTA A8							
Sunflower crop spring	21	10	7.479	0.933	1	0.933	0.09
Sunflower crop autumn	78	22	9.428	1.067	1.322	0.807	0.12
Maize crop spring	37	13	7.132	1	1.114	0.897	0.095
Maize crop autumn	37	15	9.388	0.982	1.176	0.835	0.134
Non cultivated field spring	141	34	14.224	1.281	1.531	0.836	0.066
Non cultivated field autumn	199	31	10.293	1.244	1.491	0.834	0.091
Rapeseed crop	269	38	12.076	1.086	1.58	0.688	0.129
Potato crop spring	41	12	4.927	0.833	1.041	0.8	0.184
Potato crop autumn	95	28	13.388	1.177	1.447	0.814	0.096
CULATTA							
Mixed broadleave forest spring	294	44	14.346	1.246	1.643	0.758	0.104
Mixed broadleave forest autumn	119	35	16.706	1.26	1.544	0.816	0.088
COLMATE DEL BOZZONE							
Pinewood summer	236	33	10.403	1.141	1.519	0.751	0.12
Le Lame spring	627	52	13.47	0.726	1.716	0.424	0.454
Le Lame autumn	193	37	13.596	1.239	1.568	0.79	0.098

Table 2. Number of unique species captured with pitfall traps occurring at the different sites

	unique n	total n	%
Massaciuccoli Lake autumn	24	58	41.4
Le Lame spring	16	52	30.8
Le Lame autumn	15	37	40.5
Non cultivated S21 spring	14	66	21.2
Woodland with poplars spring	14	46	30.4
Pinewood summer	11	33	33.3
Mixed broadleave forest spring	9	44	20.5
Non cultivated field spring	8	34	23.5
Mixed broadleave forest autumn	7	35	20
Non cultivated S21 autumn	7	33	21.2
Potato crop autumn	5	28	17.9
Woodland with poplars autumn	5	27	18.5
Non cultivated field autumn	4	31	12.9
Maize crop autumn	2	15	13.3
Potato crop spring	2	12	16.7
Massaciuccoli Lake spring	2	29	6.9
Sunflower crop autumn	1	22	4.5
Maize crop spring	0	13	0
Sunflower crop spring	0	10	0

Table 3. Percentage of similarity of the different species at the different study sites and on the inflorescences calculated with Renkonen's Similarity Index (f= female. m= male. n= numbers; sp = species)

		n	n sp	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
Sunflower crop spring	7	21	10	10.3	24.7	13.6	22.4	10.8	15.8	16.8	3.8	4.7	3.3	9.0	2.4	7.0	4.9	3.7	2.8	6.3	1.5
Sunflower crop autumn	8	78	22		12	21.6	12	39.5	6.3	16.3	8.5	9.6	6.7	13.5	12.2	7.3	12.2	7.9	11.4	10.9	6.7
Maize crop spring	9	37	13			16.7	35.3	12.8	13.6	17.1	5.6	9.1	6.6	0.4	4.4	11.3	4.5	5.4	5.3	10.6	4.4
Maize crop autumn	10	37	15				11.4	24.9	8	21.5	7.3	4.1	3.1	13	12	8	6.7	5.2	7.7	7.3	7.4
Non cultivated field spring	11	141	15					18.2	12.2	12.7	6.3	11.3	16.2	8	6.4	13.6	6.4	5.3	7.1	6.6	7.0
Non cultivated field autumn	12	199	15						10.3	31.5	10.3	15.8	6.4	15	12.3	11.5	12.3	8.9	9.6	9.1	7.1
Potato crop spring	13	41	15							4	1.8	2.2	4.9	4	0	4	0	1.8	0	0	1.5
Potato crop autumn	14	95	15								7.5	10.3	3.9	1	8.9	8.2	7	7.3	7.8	9.6	8.9
Mixed broadleave forest spring	15	294	15									31.6	11.6	8	16.7	16.3	14.9	14.3	16.6	12.3	6.3
Mixed broadleave forest autumn	16	119	15										11.5	11	15.3	16.9	11.5	11.0	11.6	10.7	6.9
Le Lame spring	17	627	52											3	9.1	14.6	10.3	11.4	11.1	6.6	10
Le Lame autumn	18	193	37												11.1	12.0	13	9.2	16.4	11.9	10.5
Pinewood summer	19	238	33													15.1	11.7	12.9	12.4	14.6	7.1
Non cultivated S21 spring	20	222	66														13.6	17.9	14.6	15.9	11.6
Non cultivated S21 autumn	21	160	33															14.5	12.4	14	8.3
Woodland with poplars spring	22	343	48																14.1	8.7	10.5
Woodland with poplars autumn	23	182	27																	14.3	4.9
Massaciuccoli Lake spring	24	349	58																		12.6
Massaciuccoli Lake autumn	25	248	29																		

		n	n sp	2	3	4	5	6
Inflorescences sunflower	1	4397	109	20.3	12.2	1.7	4.7	0.8
Inflorescences maize	2	906	63		11.0	1.3	4.7	1.3
Inflorescences rapeseed	3	2022	56			2.6	3.8	1.4
Inflorescences poplar m Massaciuccoli	4	58	13				23.5	3.6
Inflorescences poplar f Massaciuccoli	5	141	26					2.4
Inflorescences poplar m Fortino Nuovo	6	383	16					

>20
20/>10
10/>5
5/>2.5
2.5/<2.5

Table 4. Diversity indices calculated for the inflorescences of the experimental crops and for poplars (f= females. m= males. n= numbers. sp = species).

	Total n	sp n	Alpha	Shannon H'	Shannon H max	Pielou J'	Simpsons D
Popiar m Fortino Nuovo	383	16	3.422	0.477	1.204	0.396	0.572
Popiar m Massaciuccoli	58	13	5.206	0.786	1.114	0.688	0.267
Popiar f Massaciuccoli	141	26	9.363	1.147	1.415	0.81	0.095
Sunflower Culatta	4397	109	20.256	2.171	4.691	0.463	0.218
Maize Culatta	906	63	15.395	2.343	4.143	0.566	0.156
Rapeseed Culatta	2022	56	10.657	0.897	1.748	0.513	0.205

Table 5. Inflorescences of sunflower crops: total captures in the five sampling weeks (A) and in the three hours of the day (B).

A	Total	1	2	3	4	5
Total n	4397	549	1088	552	833	1565
	%	%	%	%	%	%
Heteroptera	53.85	9.75	11.53	6.94	7.35	21.36
Homoptera	15.83	2.52	6.00	3.12	2.98	1.21
Coleoptera	15.42	0.46	1.52	0.16	2.38	10.85
Araneae	4.62	0.66	1.64	0.77	0.71	0.84
Diptera	1.51	0.50	0.41	0.18	0.14	0.39
Hymenoptera	1.80	0.30	0.48	0.16	0.39	0.45
Lepidoptera	1.09	0.43	0.46	0.07	0.09	0.05
Neuroptera	0.75	0.14	0.14	0.05	0.16	0.27
Thysanoptera	4.78	0.66	2.75	1.05	0.20	0.11
Others	0.16	0.02	0.00	0.02	0.00	0.07

B	8.00	12.00	16.00
	%	%	%
Heteroptera	18.6	17.5	17.7
Homoptera	5.4	4.2	3.2
Coleoptera	7.1	4.8	3.5
Araneae	1.7	1.7	1.2
Diptera	0.8	0.3	0.5
Hymenoptera	0.6	0.8	0.5
Lepidoptera	0.5	0.3	0.3
Neuroptera	0.5	0.1	0.1
Thysanoptera	1.1	3.5	0.7
Others	0.1	0.1	0.1

Fig. 1. Massaciuccoli Lake; Lake system. Location of the different sampled areas (for further explanations see text)

Fig. 2. Massaciuccoli Lake; Lake system. Area with burnt Phragmites australis ("a" of Fig. 1).

Fig. 3. Massaciuccoli Lake; Anghetto channel. Internal bank with Periploca graeca ("d" of Fig. 1).

Fig. 4. Ontanelli Locality; Incolto S21. Non cultivated area with Artemisia coerulescens.

Fig. 5. Fortino Nuovo Locality; Woodland with wild poplar trees. Winter floodings.

Fig. 6. Culatta Locality; Experimental sunflower crops.

Fig. 7. Culatta Locality; Experimental maize crops.

Fig. 8. Culatta Locality; Mixed broadleave forest during the winter.

Fig. 9. Colmate del Bozzone. Molinio-Holoschoenion in spring.

Fig. 10. Colmate del Bozzone. Spring blossoming.

Fig. 11. Pitfall traps intercepting surface active macroinvertebrates.

Fig. 12. Active captures on the inflorescences of sunflowers.

Fig. 13. Bees foraging on sunflowers.

Fig. 14. Osmia sp. (Hymenoptera. Apoidea) on a rapeseed plant.

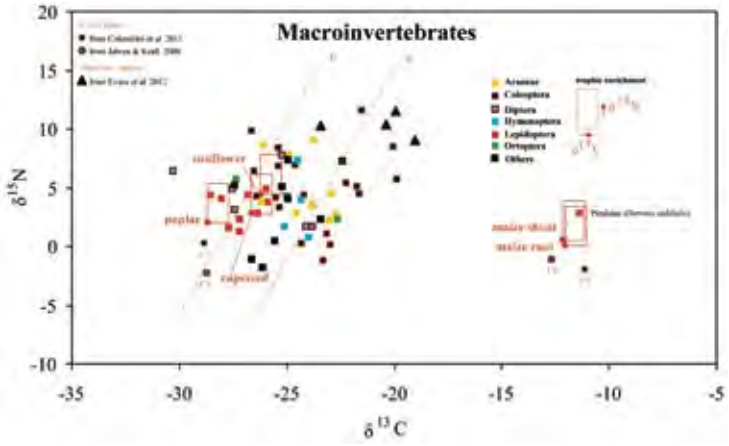

Fig. 15. Stable isotopes of δ13C and δ15N of the different plants and macroinvertebrates that were investigated (for further explanations see text).

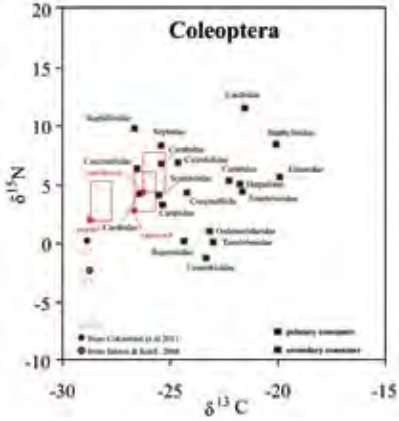

Fig. 15. Stable isotopes of δ13C and δ15N of the different Coleoptera families that were investigated (for further explanations see text).

Chapter 3

Assessment of local biodiversity

3.3 - Soil microorganisms diversity

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Introduction

Soil is the greatest reservoir of biodiversity on the planet. Soil biodiversity is mainly represented by microbial communities of bacteria (Ward 2002, Torsvik et al. 2002) and fungi (Gams 2007); one gram of soil can contain between 10^9 and 10^{11} bacterial cells (Torsvik et al. 2002, Watt et al. 2006) and up to 200 m fungal hyphae (Leake et al. 2004). Vegetation type, together with soil moisture and carbon and nutrient availability, may influence soil microbial community composition (Buckley and Schimdt 2002). Vegetation influence would take place mainly at the level of the soil volume where microorganism-mediated processes are under the influence of the plant roots, the so called rhizosphere environment. The rhizosphere concept has been defined more than 100 years ago (Hiltner 1904). Microorganisms are more abundant in the rhizosphere as compared with the corresponding bulk soil (Berg and Smalla 2009).

Up to more than 30.000 bacterial and archaeal operational taxonomic units (OTUs) were detected in the rhizosphere microbiome (Mendes 2011). Plant roots continuously modify the soil environment by rhizodeposition, a process that, by releasing a wide range of low and high-molecular weight compounds (Hinsinger et al. 2005), creates selective pressure on the local microbial communities (da Rocha et al. 2009, Koranda et al. 2011, Lambers et al. 2009, Paterson et al. 2007, Pinton et al. 2007) and shaped the composition and activity of microbial populations (da Rocha et al. 2009) in a plant species-specific manner (Berg and Smalla 2009). Conversely, belowground-living microorganisms indirectly and directly influence the productivity, diversity and composition of plant communities (van der Heijden et al. 2008).

The dynamic of soil microorganisms have important implications for the response of subsurface soil ecosystems to perturbations.

Most of our knowledge on the influence that environmental factors can exert on soil microbial diversity are the results of studies focused on cultivated herbaceous and tree plant-species of agriculture interest and only seldom wild plants have been investigated.

However, for issue of nature conservation, it is important to recognize possible modifications that human activities can produce in soil microbial communities and their interactions with wild plant in natural environments. In this respect, of particular relevance is the forecasted increases in genetically modified (GM) crops over the last decades (Carpenter 2011). Concerns have been raised that some traits carried by genetically modified plant (GMP) may negatively affect the environment and the beneficial soil biota, potentially leading to alterations in soil functioning.

Among these environmental concerns, the unintentional impact that GMPs might have on soil-associated microbes, especially rizosphere-inhabiting bacteria, is still a poorly studied and understood area (Filion 2008, Kowalchuk et al. 2003). Insecticidal proteins from transgenic crops expressing the insecticidal *Bacillus thuringiensis* protein (Bt) are exemplary of the possible impact of GMPs on soil microorganisms. Bt proteins are released by the root exudates of Bt-maize and by Bt-plant biomass during and after harvest (Saxena and Stotzky 2001) and represent a potential exposure hazard to non-target organisms (de Vaufleury et al. 2007). Further examples are toxic agents as antibiotics, herbicides or drugs produced by selectable marker genes introduced in transgenic plants together with the transgene.

Although data on their effects on microbial diversity are limited, these agents could exhibit activity against a broad range of bacteria and fungi and could potentially affect natural plant-associated bacterial and fungal populations (Miki and McHugh 2004). GMP transgenic products can directly or indirectly affect unintentional target organisms, but other risks are associated also to the use of GMP: (1) the mobilization of the transgene in the environment by pollen-mediated gene flow, from one plant into another (Hüsken et al. 2010); and (2) the horizontal gene transfer (HGT), between plant and other organisms; microorganisms are the most likely final hosts of a HGT event but also they function as intermediate hosts in the further transfer of the transgenic gene to other (prokaryotic and eukaryotic) organisms (Brigulla and Wackernagel 2010, Keese 2008). Overall, pollen-mediated gene flow and HGT are able to move in time and space the eventual toxic effects of transgenic products on natural biodiversity.

For the above reported reasons, environmental risk assessment of GM crops remains the subject of many studies (Sparrow 2010). Any evaluation of the environmental risk by GM crops must take account, among other things, of the actual biodiversity at the site where GMPs should be cultivated, before cultivation starts. As regard microbial diversity in soil, its exploration is a challenging task (Zhang and Xu 2008). In fact, more than 99% of microbial cells in soil are not cultivable by standard laboratory procedures (da Rocha et al. 2009, Van der Heijden et al. 2008). Methods that rely on direct amplification and analysis of the 16S rDNA gene allow more comprehensive sampling of microbial communities and are rapidly replacing cultivation as a way to compare their composition (type of bacteria present), richness (the number of types) and structure (the frequency distribution or relative abundance of types) (Zhang and Xu 2008).

Two main objectives of the microbial diversity study were performed:

1. the evaluation of the actual biodiversity of bacterial and fungal communities (in term of composition, richness and structure) and its seasonal variation in soils associated with superficial roots of poplar and maple trees and herbaceous plants in different sites of the Tuscany Regional Park of Migliarino-San Rossore-Massacciuccoli.
2. the identification of microbial indicator species to use in the definition of a monitoring risk index.

To evaluate microbial diversity we performed:

c. a cultural-dependent approach. In this case a dilution-plating strategy on selective plates has been used to obtain bacterial and fungal viable counts. Furthermore, a collection of isolates has been established representing the major components of cultivable microorganisms in the analyzed soils.
d. a cultural-independent approach. For this purpose, terminal restriction fragment length polymorphism (T-RFLP) analysis has been used. T-RFLP is based on amplification of a region of DNA common to multiple species using conserved PCR primers, of which one or both are fluorescently 5'-labeled. The pool of amplicons is then digested with a restriction enzyme, and based on sequence heterogeneity among species, a collection of labeled terminal fragments is produced. These are separated via capillary electrophoresis, resulting in a series of peaks that constitutes a profile of the microbial community of the soil sample. Target for T-RFLP were the 16S rDNA gene and the ITS region for bacteria and fungi, respectively, and DNA fingerprinting have been obtained that account for major microbial components from soil samples. T-RFLP profiles have been used to evaluate seasonal variability of the microbial communities from soils associated to different plants.

To identify bacterial indicators to use in risk assessments of genetically modified crops, we took advantages of the ability of T-RFLP analysis to formulate theoretically predictions on community species composition by computer simulations of the T-restriction fragment (T-RF) size (Shyu et al. 2007) available for complete bacterial gene sequences deposited in the RDP database. Only T-RFs, with suitable characteristics have been choose as indicators: (1) they must be present in a percentage of samples $\geq$ 20% of a specific group of samples (i.e., type of tree); (2) they must represent at least 1% of the community. Afterwards, the identity of T-RF indicators with bacterial species that are unwanted targets of transgenic products from GMP crops will be searched in the scientific literature.

Results and discussion

Site characteristics and soil sampling

This study was conducted in three different field sites (Table 1) located in two different Area within the Migliarino–San Rossore–Massaciuccoli Regional Park (Tuscany, Italy). The sites and the corresponding soils were classified as: (A) Site1, mixed deciduous hydric forest with Humic Eutradepts, medium-coarse, mixed, thermic soil; (B) Site 2, cultivated poplars, unknown soil; (C) Site 3, mixed deciduous meso-hydric forest with Fragic Hapludalfs, fine, mixed, thermic soil.

Table 1. Characteristics of the sampling sites.

Chemistry characteristics of soils from sampling sites are reported in Table 2 sorted by year and season.

Site	Area (m^2)	Mean distances between trees (m)	Mean distances between trees (m)	Sampled trees[a]	N° of samples	Sampling density[b]
1	7,900	1-2 (325)	51	PN	7	23.3 %
2	77,745	2-3 (8250)	7	PC	4	n.d.
3	118,000	3-1 (8310)	217	A	11	4.12 %

[a] PN, *Populus alba*; PC, *Populus nigra* x *Populus deltoides*; A, *Acer campestre* [b] percentage of sampled trees on the total number of tree from the same species in that site

Table 2. Chemistry characteristics of soil from sampling sites sorted by year and season.

Tree type	Season	Year	% of moisture (SD)[a]	pH[b]	% organic matter[b]
PN	A	2010	37.56% (0.042)	7.10	15.09
PN	W	2011	33.71% (0.022)	7.10	14.17
PN	SP	2011	33.48% (0.052)	7.10	14.42
PN	SU	2011	15.29% (0.043)	7.30	12.35
PN	A	2011	21.07% (0.033)	7.40	14.70
PN	W	2012	29.05% (0.031)	7.40	12.87
PN	SP	2012	27.11% (0.046)	7.20	16.26
PN	SU	2012	16.97% (0.056)	7.30	15.32
PC	SP	2011	17.51% (0.027)	7.70	9.51
PC	SU	2011	4.88% (0.012)	7.80	10.10
PC	A	2011	14.43% (0.010)	7.70	9.41
PC	W	2012	23.74% (0.020)	7.60	9.27
PC	SP	2012	17.95% (0.033)	7.50	10.91
PC	SU	2012	4.37% (0.017)	7.90	12.20
A	W	2011	32.08% (0.059)	7.20	15.69
A	SP	2011	21.95% (0.045)	6.60	16.99
A	SU	2011	15.14% (0.039)	7.20	15.81
A	A	2011	16.90% (0.034)	6.80	14.83
A	W	2012	25.84% (0.058)	7.00	14.03
A	SP	2012	24.93% (0.079)	7.00	14.81
A	SU	2012	12.51% (0.033)	7.00	15.98
BULK	SU	2012	n.d.	8.00	7.65
C	SU	2012	n.d.	7.90	8.21
M	SU	2012	n.d.	7.90	8.10

[a] Moisture values have been determined individually on all soil samples and reported as mean seasonal values; in brackets are the standard deviation values.

[b] Reported are the values, determined by the Loss on Ignition Method, of pools of soils from the same season.

Seven natural poplars (*Populus alba*: P1, P2, P3, P4, P5, P6, P11) in site 1, four cultivated poplars (hybrid "Triplo" clone *Populus nigra* x *Populus deltoides*: PC1, PC2, PC3, PC4) in site 2 and eleven maples (*Acer campestre*: A9, A13, A104, A112, A119, A142, A182, A204, A249, A260) in site 3, were collected seasonally from Autumn 2010 to Summer 2012. Soil samples were collected by a bulb planter (10 cm wide x 15 cm depth) at a distance of around 20 cm from the tree trunk, placed in a sterile plastic bag and immediately stored at 5°C; the same day, the samples were brought to the lab where they were sequentially sieved through 5 mm and 2 mm pore size stainless steel sieves. Sieved soils were split into aliquots that were stored at 4 °C for total microbial counts, moisture content, pH determination, loss on ignition measure, and at -80 °C for molecular analysis.

Tables 3 and 4 report the type and number of soil samples collected during the whole

project and sorted by year and season; moreover, the type of analysis done on each sample is reported.

Table 3. Origin and number and soils samples, sorted by year and season, from different sites and performed analysis.

Area	Site	Year	Season[a]	Samples			Performed analysis		
				Natural poplars (PN)	Cultivated poplars (PC	Maples (A)	Cultural	Molecular	Soil chemistry
AREA 2	1 (Fortino nuovo)	2010	A	7	-	–	✓	✓	✓
		2011	W	7	-	–	✓	✓	✓
		2011	SP	7	-	–	✓	✓	✓
		2011	SU	7	-	–	✓	✓	✓
		2011	A	7	-	–	✓	✓	✓
		2012	W	7	-	–	✓	✓	✓
		2012	SP	7	-	2[b]	✓	✓	✓
		2012	SU	7	-	–	✓	✓	✓
	2 (Fortino nuovo)	2011	W	-	4	–	✓	✓	✓
		2011	SP	-	4	–	✓	✓	✓
		2011	SU	-	4	–	✓	✓	✓
		2011	A	-	4	–	✓	✓	✓
		2012	W	-	4	–		✓	✓
		2012	SP	-	4	–		✓	✓
		2012	SU	-	4	–		✓	✓
AREA 3	3 (Culatta)	2011	SP	-	-	11		✓	✓
		2011	SU	-	-	11		✓	✓
		2011	A	-	-	11		✓	✓
		2012	W	-	-	11		✓	✓
		2012	SP	-	-	11		✓	✓
		2012	SU	2[b]	-	11		✓	✓

[a] A, Autumn; W, Winter; SP, Spring; SU, Summer; [b] Controls

Table 4. Origin and number of soils samples from herbaceous plants and performed analysis.

Area	Site	Year	Season[a]	Samples			Performed Analysis		
				Bulk soil	*Brassica napus* (C)	*Synapis arvensis* (M)	Cultural	Molecular	Soil chemistry
AREA 3	4 (Culatta)	2012	SU	2[b]	7	13		✓	✓

[a] SU, Summer; [b] Controls

Seasonal variation of soil microbial communities within each sampling site

Table 5 reports the numbers of microbial strains (bacteria and fungi) isolated from viable count plates during the whole project and preserved as pure-cultures to form a collection of strains. Bacterial strains are kept at -80°C in a 20% glycerol media, fungi strains are kept at 4°C in distilled water. For each isolate a description of colony and cellular morphology has been done and reported in a database with a hyperlink to a picture of the colony. The pure-culture collection is *representatives of the major* colony types of *aerobic heterotrophic cultivable* bacteria in the sampled soils.

Table 5. Number of pure-culture bacterial and fungal strains and their origin.

Type of tree	Bacteria	Fungi
PN	194	177
PC	39	37
A	153	138
TOTAL	386	352

Following are the data related to different sites in different area of the Park where the soil sampling for microbial characterization were performed. For each site are reported: (1) viable bacterial and fungal counts obtained by plating on selective media; (2) bacterial and fungal diversity (expressed as Richness, Evenness and Shannon's index) as assessed by Terminal Restriction Length Polymorphism (T-RFLP) analysis; (3) clustering analysis of bacterial and fungal soil communities; (4) correspondence analysis (CA) for bacterial and fungal communities; (5) heat-map and Bray-Curtis distances between groups of samples representing bacterial and fungal communities.

AREA 2-SITE 1 (FORTINO NUOVO), NATURAL POPLARS SAMPLING

Bacterial viable counts from natural poplars in Site 1 are always at least two order of magnitude higher than fungi ones. There is no clear evidence of seasonal influence on microbial viable counts (Fig. 1).

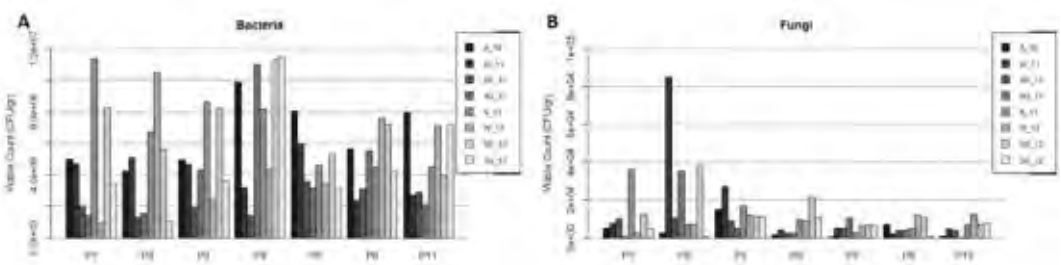

Fig. 1. A. Seasonal mean values of bacterial viable counts in soil samples from natural poplars in Site **1. B.** Seasonal mean values of fungal viable counts in soil samples from Natural Poplars in Site. Seasons are reported following the legend in table 3; numbers refers to the year of sampling.

As can be seen in Table 6, bacterial and fungal community richness values, that is the number of T-RFs in each microbial community profile, where each T-RF ideally corresponds to a distinct Operational Taxonomic Unit (OTU), vary from season to season but do not follow a seasonal pattern. Relative peak area, as a measure of species abundance, is needed to calculate Evenness and Shannon index. Evenness value of bacteria and fungi communities are high, the fungi ones higher than bacterial ones, and are not influenced by seasonality. Shannon's index account for both community Richness and Evenness, so is a good synthetic diversity index. In our case, Shannon index values describe medium-high diverse bacteria and fungi communities. The narrow range of variation of the Shannon's index values among different seasons suggests that seasonality has only low effects on microbial communities diversity although, conceivably, communities are structurally

different.

Table 6. Diversity analysis of bacterial and fungal communities in soil samples from natural poplars in Site 1. Richness, Evenness, and Shannon index have been calculated by T-RFLP data analysis. Data reported are mean values from seasonal soil samples.

	Bacteria			Fungi		
	Richness	Evenness	Shannon	Richness	Evenness	Shannon
A_10	19	0.80	2.33	18	0.83	2.65
W_11	23	0.85	2.63	17	0.91	2.57
SP_11	23	0.84	2.60	14	0.86	2.25
SU_11	23	0.81	2.54	13	0.89	2.23
A_11	24	0.83	2.65	13	0.92	2.31
W_12	26	0.83	2.70	22	0.93	2.87
SP_12	26	0.80	2.60	24	0.93	2.91
SU_12	18	0.83	2.35	18	0.93	2.62

Clustering (Fig. 2) and CA (Fig. 3) do not show any particular ordination of the natural poplars samples. In fact there is neither a temporal based (per season per year) nor an individual based (per the same tree from different seasons) ordination of the samples. Moreover, microbial communities associated with control maples do not form a distinct group, suggesting that, in the shaping of the structure of microbial communities, tree type differences are not predominant. In CA analysis, winter samples grouped, showing lower variability than other samples.

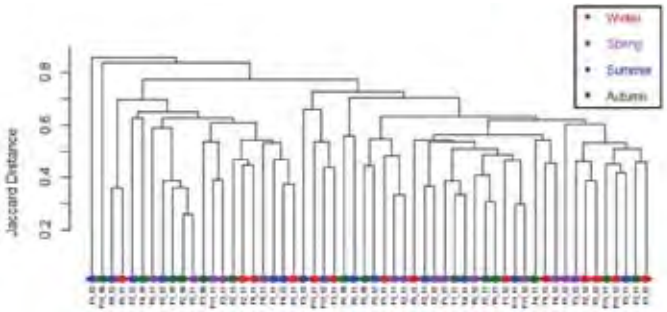

Fig. 2. UPGMA clustering of bacterial communities in soil sample from natural poplars in Site 1.

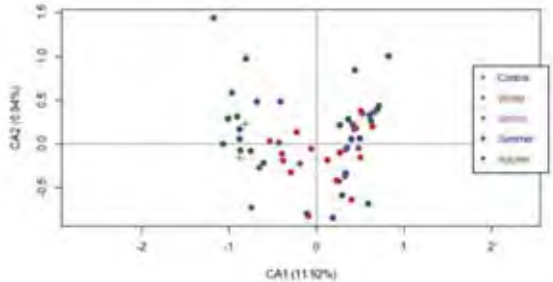

Fig. 3. Correspondence Analysis (CA) of bacterial communities in soil samples from natural poplars in Site 1 . Symbol + indicates maples sampled as controls in the same site.

Clustering analysis (Fig. 4) of fungal communities shows an ordination that is influenced by individual tree (same tree in different seasons) but not by season or year. CA ordination plot (Fig. 5) confirms this observation. Control maples, as seen for the microbial communities, do not cluster together.

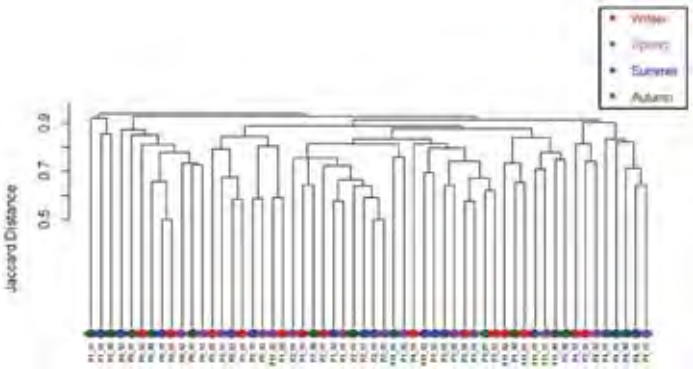

Fig. 4. UPGMA clustering of fungal communities in soil sample from natural poplars in Site 1.

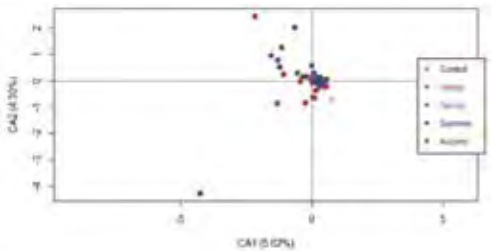

Fig. 5. Correspondence Analysis (CA) of fungal communities in soil samples from natural poplars in Site 1. Symbol + indicates maples sampled as controls in the same site.

AREA 2-SITE 2 (FORTINO NUOVO), CULTIVATED POPLARS SAMPLING.

Bacterial viable counts are always at least two order of magnitude higher than fungi ones. There is no clear evidence of seasonal influence on microbial abundance (Fig. 6).

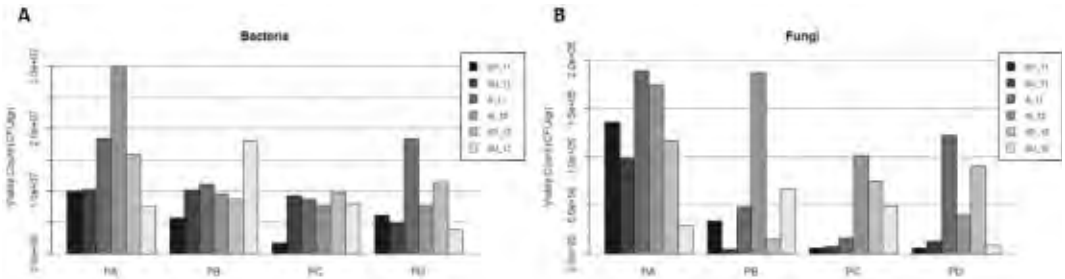

Fig. 6. A. Seasonal mean values of bacterial viable count in the soil sample from cultivated poplars in Site 2. **B.** Seasonal mean values of fungal viable count in the soil samples from cultivated poplars in Site 2. For season identification see legend of Fig. 1.

As can be seen in Table 7, bacterial and fungal community richness values, as already

observed for natural poplar in Site 1, vary from season to season but do not follow a seasonal pattern. Fungal communities show a range of richness variation wider than bacterial ones. Evenness value of bacteria and fungi communities are high, higher in fungi, and are not influenced by seasonality. Shannon index values describe medium-high diverse bacteria and fungi communities with a narrow range of seasonal variation, suggesting that seasonality, similarly to natural poplar in Site 1, has only low effects on microbial communities diversity.

Table 7. Diversity analysis of bacterial and fungal communities in soil samples from cultivated poplars in Site 2. Richness, Evenness, and Shannon index have been calculated by T-RFLP data analysis. Data reported are mean values from seasonal soil samples.

	Bacteria			Fungi		
	Richness	**Evenness**	**Shannon**	**Richness**	**Evenness**	**Shannon**
SP_11	18	0.75	2.16	12	0.91	2.27
SU_11	17	0.81	2.27	14	0.91	2.35
A_11	20	0.79	2.35	10	0.95	2.20
W_12	20	0.84	2.52	17	0.91	2.53
SP_12	18	0.81	2.30	13	0.89	2.27
SU_12	20	0.81	2.40	14	0.90	2.30
SP_11	18	0.75	2.16	12	0.91	2.27
SU_11	17	0.81	2.27	14	0.91	2.35

Clustering analysis (Fig. 7) shows a clear, but not exclusive, temporal based ordination for samples from Summer 2012 and Spring 2011. A third bigger cluster (the rightmost one) has a mixed composition that includes all the Winter 2012, Summer 2011 and Spring 2012 samples. Autumn 2011 samples are scattered in these last three clusters. The same divisions are well visible in the CA ordination plot (Fig. 8), suggesting that, at least in part, bacterial communities composition in cultivated poplars samples is influenced by seasonality.

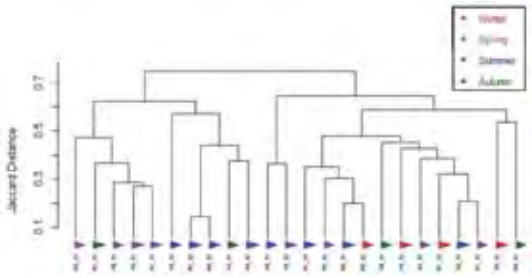

Fig. 7. UPGMA clustering of bacterial communities in soil samples from cultivated poplars in Site 2.

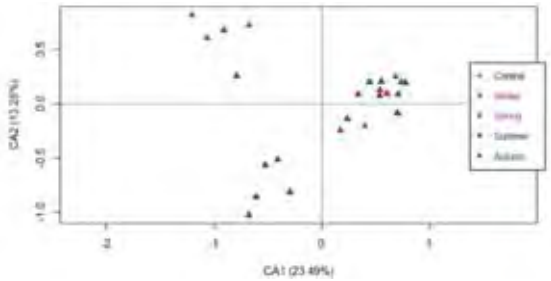

Fig. 8. Correspondence Analysis (CA) of bacterial communities in soil samples from cultivated poplars in Site 2.

Clustering (Fig. 9) and CA (Fig. 10) do not show any specific ordination of the samples. In fact, there is neither a temporal based (per season and year) nor an individual based (per the same tree from different seasons) ordination of the samples.

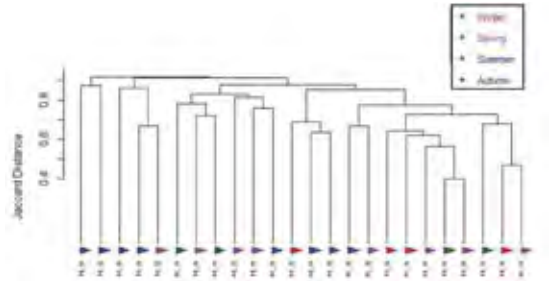

Fig. 9. UPGMA clustering of fungal communities in soil samples from cultivated poplars in Site 2.

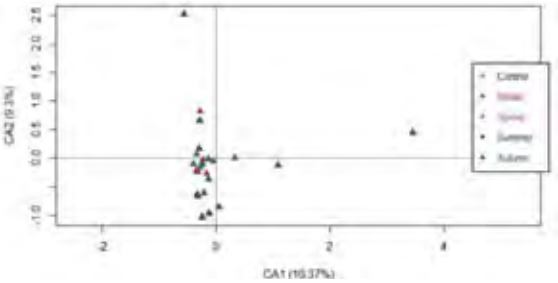

Fig. 10. Correspondence Analysis (CA) of fungal communities in soil samples from cultivated poplars in Site 2.

AREA 3-SITE 3 (CULATTA), MAPLES SAMPLING.

Bacterial viable counts from maples in Site 3 are always at least two order of magnitude higher than fungi ones. There is no clear evidence of seasonal influence on microbial viable counts (Fig. 11).

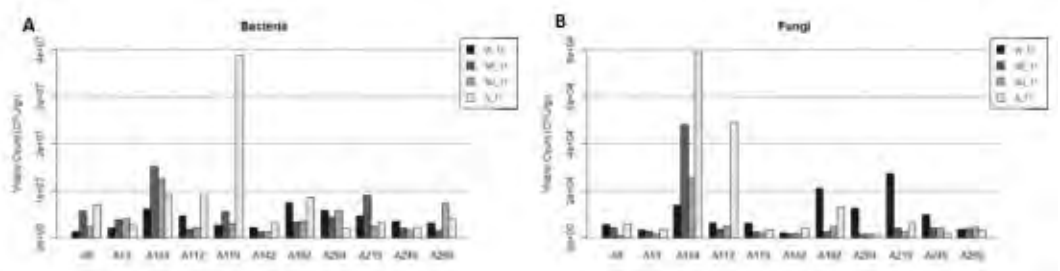

Fig. 11. A. Seasonal mean values of bacterial viable counts in soil samples from maples in Site 3. **B.** Seasonal mean values of fungal viable counts in soil samples from maples in Site 3. For season identification see Fig. 1 legend.

As can be seen in Table 8, bacterial and fungal community richness values, as already observed for natural and cultivated poplars, vary from season to season but do not follow a seasonal pattern. Differently from poplars samples, fungal communities in maples show a range of richness variation narrower than bacterial ones. Evenness value of bacteria and fungi communities are high, higher in fungi, and are not influenced by seasonality. Shannon index values describe medium-high diverse bacteria and fungi communities with a narrow range of seasonal variation, suggesting that seasonality, similarly to natural and cultivated poplars, has only low effects on microbial communities diversity. Differently from poplars, bacterial and fungal values show similar patterns of variation from season to season.

Table 8. Diversity analysis of bacterial and fungal communities in soil samples from maples in Site 3. Richness, Evenness, and Shannon index have been calculated by T-RFLP data analysis. Data reported are mean values from seasonal soil samples.

	Bacteria			Fungi		
	Richness	**Evenness**	**Shannon**	**Richness**	**Evenness**	**Shannon**
W_11	15	0.80	2.16	14	0.93	2.42
SP_11	19	0.77	2.27	12	0.91	2.16
SU_11	25	0.79	2.55	15	0.92	2.46
A_11	21	0.79	2.40	13	0.91	2.27
W_12	27	0.82	2.69	18	0.90	2.52
SP_12	28	0.82	2.71	22	0.92	2.81
SU_12	19	0.77	2.26	14	0.86	2.23
W_11	15	0.80	2.16	14	0.93	2.42

In Clustering analysis (Fig. 12) a clear cluster of samples from Winter 2011 is visible. Moreover, other samples show a tendency to group together on the basis of the year of sampling; for instance, all samples from Summer 2012 clustered together with most samples from Spring 2011. CA (Fig. 13) also shows a distinct ordination of samples from

winter 2011 (not specified), with a great variance along the second component. Control samples from poplars in the same site do not appear to differ substantially from the other samples.

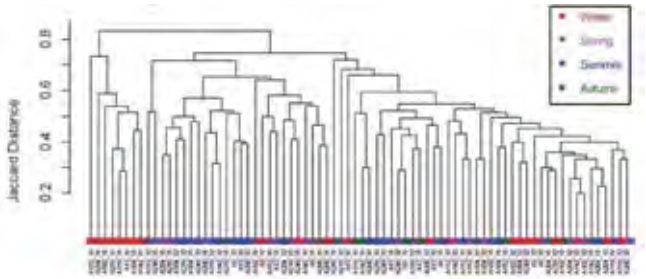

Fig. 12. UPGMA clustering of bacterial communities in soil sample from maples in Site 3.

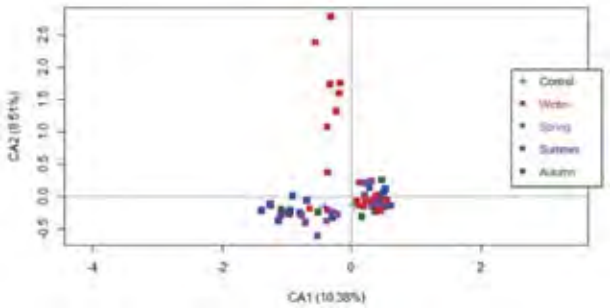

Fig. 13. Correspondence Analysis (CA) of bacterial communities in soil samples from maples in Site 3. Symbol + indicates poplars sampled as controls in the same site.

Fig. 14 show a tendency of the fungal communities to form cluster of samples from the same tree irrespective of the season of sampling. CA (Fig. 15) do not show any particular ordination of the samples.

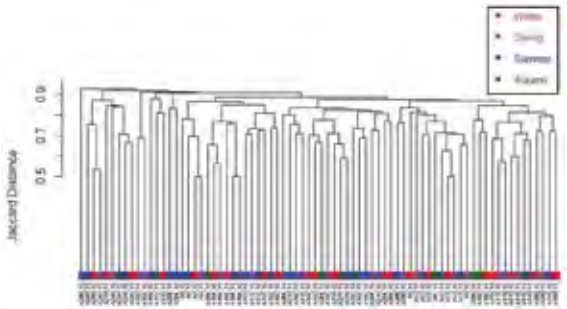

Fig. 14. UPGMA clustering of bacterial communities in soil sample from maples in Site 3.

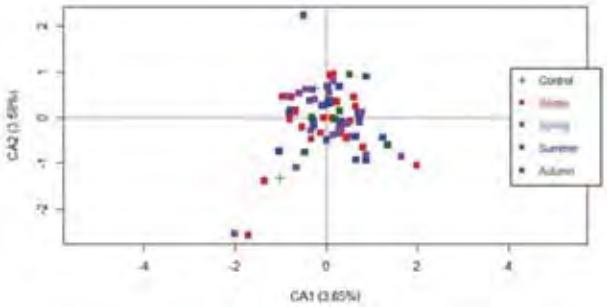

Fig. 15. Correspondence Analysis (CA) of fungal communities in soil samples from maples in Site 3. Symbol + indicates poplars sampled as controls in the same site.

AREA 3-SITE 4 (CULATTA-PARCELLA A6), HERBACEOUS PLANTS SAMPLING.
As can be seen in Table 9, bacterial communities Richness and Shannon's index values in soils from herbaceous plants are higher than in bulk soils. Conversely, fungal communities from herbaceous plants and bulk soils show more similar Richness values, but Shannon's index values in bulk soil is lower.

Table 9. Diversity analysis of bacterial and fungal communities in soil samples from herbaceous plants and in bulk soils in Site 4. Richness, Evenness, and Shannon index have been calculated by T-RFLP data analysis.

	Bacteria			Fungi		
	Richness	Evenness	Shannon	Richness	Evenness	Shannon
BULK	9	0.86	1.84	15	0.89	2.36
C	18	0.81	2.31	16	0.93	2.56
M	15	0.83	2.21	17	0.93	2.58

Clustering (Fig. 16) and CA (Fig. 17) show two clusters, one containing only but not all the *B. napus* soils from July 2012 sampling and the two bulk soil samples, the second one is broader and contains three smaller clusters: one containing only *B. napus* samples and one the majority of the *Sinapis arvensis* samples.

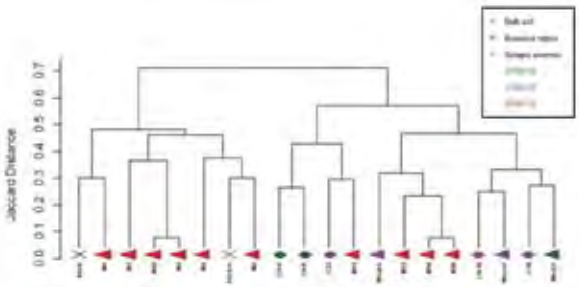

Fig. 16. *UPGMA clustering of bacterial communities in soil samples from herbaceous plants and bulk soils in Site 4.*

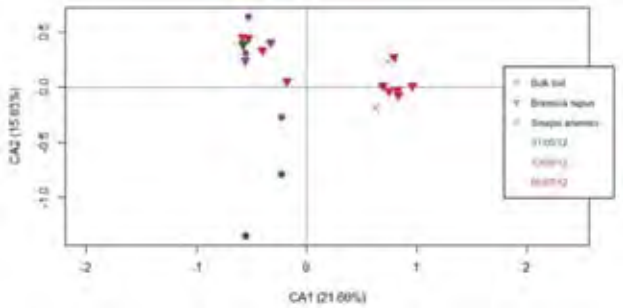

Fig. 17. Correspondence Analysis (CA) of bacterial communities in soil samples from herbaceous plants and bulk soils in Site 4.

Fig. 18 shows how bacterial communities from soil of *S. arvensis* and *B. napus* are different from each other and from those of bulk soil, with *B. napus* showing the greater difference.

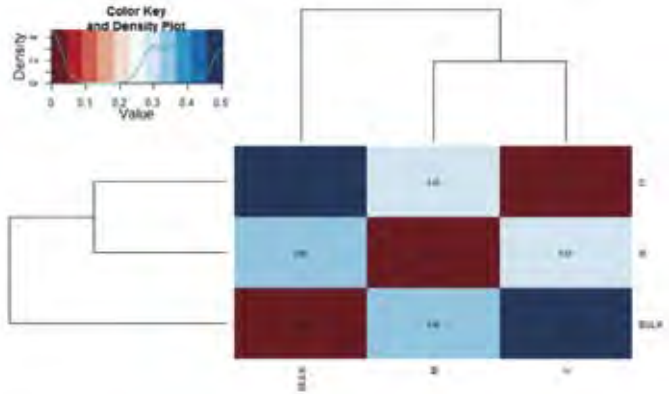

Fig. 18. Heat-map representing Bray-Curtis distances between bacterial communities profiles from soils associated to herbaceous plants and bulk soils sampled (C, B. napus; M, S. arvensis; BULK, bulk soil). Profiles are obtained as frequency of occurrence of each T-RF in each group of profiles (C,M or BULK).

Clustering (Fig. 19) and CA (Fig. 20) do not show any particular ordination of the samples.

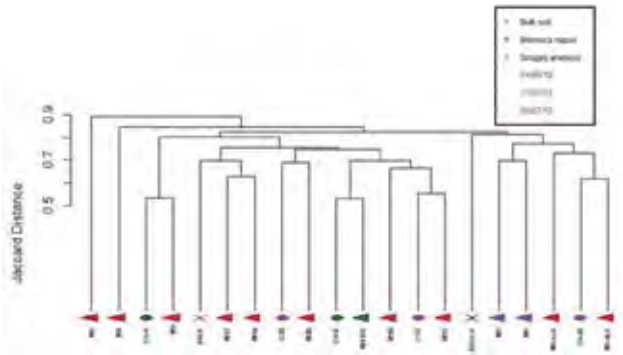

Fig. 19. UPGMA clustering of fungal communities in soil samples from herbaceous plants and bulk soils in Site 4.

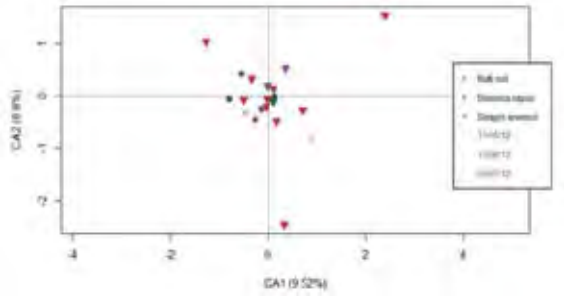

Fig. 20. Correspondence Analysis (CA) of fungal communities in soil samples from herbaceous plants and bulk soils in Site 4.

Fig. 21 shows how bacterial communities from soils of *S. arvensis* and *B. napus* are different from each other and, in a comparable way, from those of bulk soils.

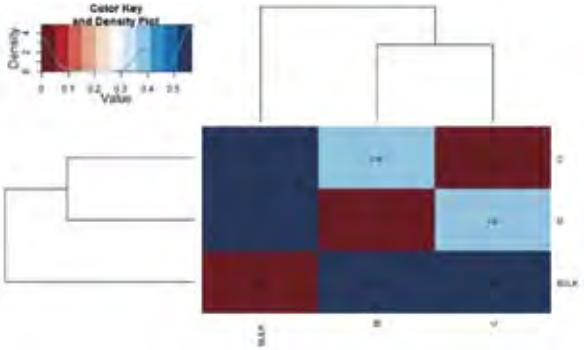

Fig. 21. Heat-map representing Bray-Curtis distances between fungal communities profiles from herbaceous plants and bulk soils samples (C, B. napus; M, S. arvensis; BULK, bulk soil). Profiles are obtained as frequency of occurrence of each T-RF in each group of profiles (C,M or BULK).

COMPARISON BETWEEN SITES

After reporting the data concerning the analysis of microbial diversity in the different sampling sites, here we report some data concerning the comparison between sites. This comparison is important because it allows to evaluate differences between communities that are due to specific components such as the type of soil or the type of tree from which the communities are from.

Fig. 22 shows a clear clustering of samples based on soil/tree type (A, PN, and PC). Bacterial communities profiles in soils from cultivated poplars in site 2 are highly diverse respect to communities from maples and natural poplars, the great diversity is observed with maples. Maples and natural poplars are more similar each other. Moreover, looking at the intra-group variability, community profiles from maple and natural poplars soils in different seasons are less diverse than intra-cultivated poplars ones. These observations could suggest that cultivation regimen affect the structure of bacterial communities.

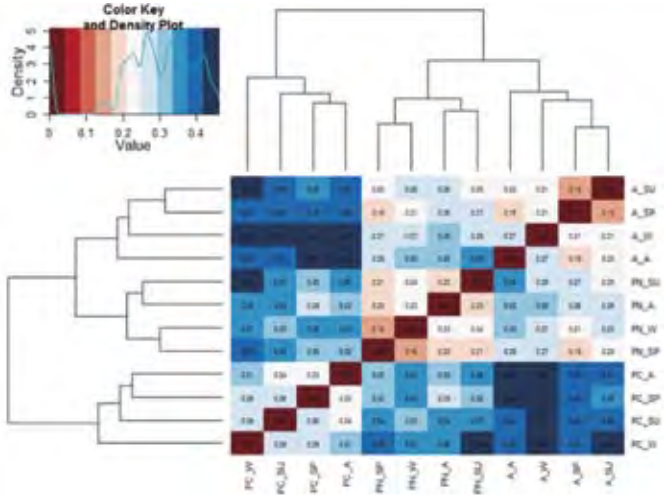

Fig. 22. Heat-map representing Bray-Curtis distances between bacterial communities profiles of soils from different sites and then from different trees (A, Maples; PN, Natural Poplars; PC, Cultivated Poplar). Profiles are obtained as frequency of occurrence of each T-RF in each group of profiles (A,PN or PC).

Fig. 23 shows a clear clustering of samples based on soil/tree type (A, PN, and PC). Like bacterial communities, fungal communities profiles from soils of cultivated poplars are highly diverse respect to those from maples and natural poplars. Overall the level of fungal diversity is higher. Moreover, looking at the intra-group variability, profiles from cultivated poplars in different seasons are more variable than those from natural poplars and comparable to those between maples. These observations suggest that, as already seen for bacterial communities, cultivation affect the structure of fungal communities too.

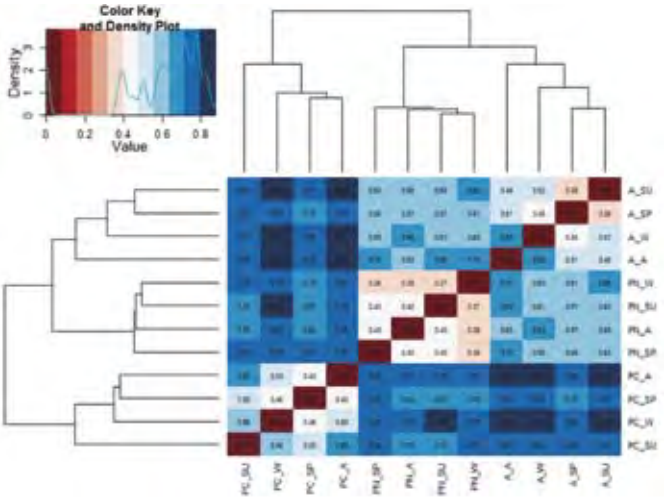

Fig. 23. Heat-map representing Bray-Curtis distances between fungal communities profiles of soils from different sites and then from different trees (A, Maples; PN, Natural Poplars; PC, Cultivated Poplar). Profiles are obtained as frequency of occurrence of each T-RF in each group of profiles (A,PN or PC).

IDENTIFICATION OF SUITABLE INDICATORS

To identify bacterial indicators to use in risk assessments of GM crops we chosen those T-RFs which are present in a percentage of samples ≥ 20% of a specific group of samples (i.e., type of tree) and that represent at least 1% of the community. These T-RF indicators and the corresponding microbial species are reported in Table 10.

Table 10. List of T-RFs that have been proposed as suitable indicators in risk assessment of GM crops and the corresponding theoretically predicted bacterial species.

T-RF LENGHT	Mica IDENTIFICATION	PC		PN		A	
		Frequence of occurrence	Relative Abundance (%)	Frequence of occurrance	Relative abundance (%)	Frequence of occurrance	Relative abundance (%)
[illegible]	*unidentified*	0.54	3.24	0.46	5.54	0.51	4.06
[illegible]	*unidentified*	0.79	1.46	0.77	1.94	0.88	3.84
105	*Ochrobactrum anthropi* *Rhizobium sp.*	not present	-	0.91	0.13	0.08	0.15
[illegible]	*Xanthobacter autotrophicus*	1.00	2.12	0.64	2.25	0.60	2.52
[illegible]	*Dinoroseobacter shibae* *Janthinobacterium sp.* *Parvibaculum lavamentivorans* *Rhodobacter sphaeroides Sphingopyxis sp.*	0.54	0.86	not present	-	0.01	0.02
162	*unidentified*	0.88	2.23	0.63	1.28	0.16	0.38
166	*Bacillus cereus*	not present	-	0.50	1.62	0.01	0.04
[illegible]	*unidentified*	0.25	0.57	0.05	0.75	not present	-
[illegible]	*Rhodobacter sphaeroides* *Sphingomonas sp.*	0.54	11.93	0.57	12.85	0.55	11.27
[illegible]	*Acidocella sp.*	0.96	16.56	0.38	6.54	0.48	11.70
[illegible]	*Polaromonas sp.* *Roseiflexus sp.* *Variovorax sp.*	0.50	1.79	0.54	2.88	0.60	2.98
[illegible]	*Rhodoferax ferrireducens*	1.00	10.74	0.82	10.05	0.69	4.66
[illegible]	*Dehalococcoides sp.* *Frankia sp.*	0.29	0.55	0.02	0.03	not present	-
[illegible]	*Arthrobacter sp.* *Clostridium beijerinckii* *Kitasatospora putterlickiae* *Kribbella jejuensis* *naphthalene-utilizing bacterium* *Slackia heliotrinireducens* *soil actinomycete* *Streptomyces sp.*	0.67	2.63	0.63	3.97	0.77	8.94
[illegible]	*Agromyces sp.* *Arthrobacter sp.* *Clostridium phytofermentans* *Psaudomonas sp.* *Thermoanaerobacter sp.*	0.67	7.56	0.73	7.00	0.77	5.52
[illegible]	*Exiguobacterium sibiricum*	0.63	16.07	0.63	8.53	0.79	8.70
472	*Alcaligenaceae bacterium* *Brevibacillus sp.* *Burkholderia sp.* *Cupriavidus necator*	0.42	1.53	0.27	1.99	0.09	0.50
[illegible]	*Stenotrophomonas maltophilia*	0.50	19.38	0.61	28.78	0.60	32.76
[illegible]	*Bacillus sp.* *Myxococcus sp.* *Paenibacillus rhizosphaerae* *Staphylococcus aureus*	0.38	0.77	0.48	3.90	0.45	1.97

Color code: red, ubiquitary; yellow, PN exclusive; dark green, PC exclusive; light green PN+PC exclusive

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Chapter 4

Delineation of an analysis method for assessing the risks of genetically modified plants on the environment

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Introduction

Definition of a risk assessment method

Recent developments and applications of biotechnology, especially genetic engineering, have revolutionized crop and plants improvement and increased the availability of valuable new traits, such as the rapid development of transgenic insect-resistant plants has brought great economic benefits and has led to a significant reduction in the usage of chemical insecticides. However, the introduction of transgenic plants into agricultural ecosystems has introduced putative ecological risks (Li et al. 2012). For these reasons, the controversy over genetically modified (GM) plants continues to be reported in the news and scientific papers. Proponents of GM crops site benefits that include increased yield, nutrient enrichment, and resistance to pests and diseases. Opponents site risks that include uncertainties regarding food safety, contamination of native crop species with genetic elements from GM crops, and danger to biodiversity (de Jesus et al. 2006).
Due to the potential environmental impacts of GM plants, there is an urgent need of define a risk method to evaluate and assess their negative impact on the environment. These assessments allow us to define predictive measures to mitigate or avoid the adverse effects that could result from potential or identified hazards (de Jesus et al. 2006).

Detection of possible risks caused by genetically modified organisms (GMOs)

According to the Ecological Society of America (ESA) possible risks of GMOs could include: (1) creating new or more vigorous pests and pathogens; (2) exacerbating the effects of existing pests through hybridization with related transgenic organisms; (3) harm to non-target species, such as soil organisms, non-pest insects, birds, and other animals; (4) disruption of biotic communities, including agro-ecosystems; and (5) irreparable loss

or changes in species diversity or genetic diversity within species (Snow et al. 2005). The complexity of ecological systems presents considerable challenges for experiments to assess the risks and benefits and inevitable uncertainties of genetically engineered plants (Wolfenbarger and Phifer 2000).

The release of GMOs highlights the general difficulty in predicting the occurrence and extent of long-term environmental effects when non-native organisms are introduced into ecosystems. Genetic modifications, through traditional breeding or genetic engineering, of crop or other species can potentially create changes that enhance an organism's ability to become an invasive species. Potential ecological impacts through invasiveness depend on existing opportunities for unintended establishment, persistence, and gene flow of an introduced organism; each of these, in turn, depends on various components of survival and reproduction of an organism or its hybrids (Wolfenbarger and Phifer 2000).

Large areas of cultivation may increase the opportunity for range overlap with compatible relatives; therefore, the probability that crop genes, newly introduced through genetic engineering or through other, more traditional techniques, will introgress into wild relatives may increase as particular cultivars are more widely adopted (Wolfenbarger and Phifer 2000).

Genetic modifications could change the propensity of outcrossing (Bergelson et al. 1998), although this has not been reported in the one crop species studied (Hokanson et al. 1997). Ecological impacts of pollen transfer, a reproductive mechanism through which introgression might occur, depend on whether hybrids survive and reproduce. Equivocal rates of survival or reproduction between transgenics and controls suggest, but do not indicate, the opportunity for introgression of transgenes into natural populations (Snow et al. 2005), depending on subsequent gene flow and selective pressures. Not all studies support these conclusions (Lefol et al. 1996), and ecological consequences in non-agricultural habitats and ecosystems largely remain unstudied (Wolfenbarger and Phifer 2000).

Potential benefits

Reduced environmental impacts from pesticides. As regulations are considered, the potential risks of GMOs should be evaluated and compared to possible environmental benefits, as well as to risks from conventional and other agricultural practices, such as organic farming. For example, insect-resistant and herbicide-tolerant transgenic crops may decrease the use of environmentally harmful chemicals to control pests (Wolfenbarger and Phifer 2000). The shift to post emergent control of weeds may promote no-till and conservation tillage practices that can decrease soil erosion and water loss and increase soil organic matter (Cannel et al. 1994). Studies are needed to address whether soils are improving as a result of crops genetically engineered for herbicide tolerance.

Moreover, if genetically engineered crops increase yields, some suggest that environmental benefits will include the preservation of natural habitats because less land may be developed for agriculture. However, the potential environmental benefits of genetically

engineered crops through increased yield may be greatest in developing countries where agricultural output may stand for the most improvement (Wolfenbarger and Phifer 2000). Transgenic plants can increase removal of toxic heavy metals from polluted soils and waters and sequester these into plant tissue available for harvest (Zhu et al. 1999), or can transform pollutants into less toxic forms (Bizily et al. 2000).
Environmental remediation through transgenic plants has not yet been used widely, so net environmental benefits have not been measured (Wolfenbarger and Phifer 2000).

Problems related to definition of Risk Assessment method

Environmental and cultivar variability complicates the task of assessing risk. Ecosystems are complex, and not every risk associated with the release of new organisms, including transgenics, can be identified, much less considered. Unknown risks may surface as the frequency and scale of the introduction increases (Levin et al. 1989). Because some consequences, such as the probability of gene flow, are a function of the spatial scale of the introduction (Klinger et al. 1992). Ecological relationships include many cascading and higher order interactions that are intrinsically difficult to test and evaluate for significance at limited temporal and spatial scales. At larger spatial scales, there is a greater possibility for contact with sensitive species or habitats or for landscape-level changes because at larger scales more ecosystems could be altered (Snow et al. 2005). Transgenic organisms, such as genetically engineered crops, released into the environment will potentially interact with a diversity of habitats in time and in space, and the potential risks from a single type of transgenic organism may vary accordingly. Risk assessments will need to be especially sensitive to temporal and spatial factors (Wolfenbarger and Phifer 2000). Rigorous, well-designed studies of the benefits and risks associated with GMOs are needed. Ecologists, evolutionary biologists, and a wide range of other disciplinary specialists should become more actively involved in research aimed at quantifying benefits and risks posed by GMOs in the environment. Because of the inherent complexity of ecological systems, this research should be carried out over a range of spatial and temporal scales (Snow et al. 2005).

Assessing the environmental risks of GMOs

Risk assessment is widely used in decision-making concerning the release of genetically modified (GM) plants into the environment (EFSA 2010). Enviromental Risk Assesment (ERA) is used in decision-making for approval of a GM organism (Fitz Patrik et al. 2009). Possible influences of the plant and practices related to its cultivation have to been evaluated all and this evaluation is performed by risk analysis (de Jesus et al. 2006) The purpose of risk assessment is to aid decision making, not to increase scientific knowledge per se (Hill and Sendashonga 2003, Romeis 2009). Requirements for new data for risk assessment should therefore be limited to those necessary to reach a confident conclusion of acceptable risk. If suitable data on which to base a regulatory decision are already available, the temptation to request additional data to answer interesting scientific questions should be resisted (Raybould 2007): risk assessments should be based on ‘need to know’ not ‘nice

to know' data. A broad consensus had developed that there were potential environmental risks of transgenic plants requiring assessment and that this assessment must be done on a case-by-case basis, taking into account the transgene, recipient organism, intended environment of release, and the frequency and scale of the intended introduction (Andow and Zwahlen 2006). Risk analysis follows a structured approach with three distinct but closely related steps: risk assessment, risk management, and risk communication (de Jesus et al. 2006).

Background: EFSA guidelines on the environmental risk assessment (EFSA, 2010)

Environmental risk assessments can provide high confidence of minimal risk by testing theories, "risk hypotheses", that predict the likelihood of unacceptable harmful events. The creation of risk hypotheses and a plan to test them is called problem formulation. Effective problem formulation seeks to maximize the possibility of detecting effects that indicate potential risk; if such effects are not detected, minimal risk is indicated with high confidence (Raybould 2006). According to EFSA guidelines (2010), the risk assessment follows six steps (1) problem formulation including hazard identification; (2) hazard characterisation; (3) exposure characterisation; (4) risk characterisation; (5) risk management strategies; and (6) an overall risk evaluation.

Step 1: Problem formulation including hazard identification.

The problem context for risk assessment reflects values derived from the broad environmental policies and goals that direct risk analysis. Establishing the problem context sets the parameters for the risk assessment, including; protection goals, environmental scope, standard assessment endpoints (Suter 2000), and assessment methodology. The process of integrating the likelihood and consequences of exposure, in terms of harm, forms the basis of environmental risk assessment (ERA). As the first step in ERA, the problem formulation (PF) establishes the parameters that are of greatest relevance to the assessment. An inadequate PF may compromise the entire ERA and add to the level of uncertainty in subsequent decision-making. Frequent outcomes of this type of failure are continuing requests for more data, disproportionate risk mitigation measures and miscommunication of risk findings; this results in increased concerns about the environmental impact (Johnson et al. 2007, Raybould 2006) and leads to delayed decision-making. Some authors contend that such delays may lead to increased negative environmental impacts because of the consequent delays in the introduction of environmentally beneficial products (Raybould 2006, 2007). Additionally, an ERA with a poorly developed PF may have inadequately specified or inappropriate expressions of the environmental value to be protected (benefits including processes by which the environment produces resources), or insufficient clarity regarding the purpose and use of the data being collected. This report presents a framework for constructing PFs that can be applied to ERAs for GM plants (Wolt et al. 2010).

Step 2: Hazard characterization.

Hazard characterization in this Guidance Document (GD) is defined as the qualitative and/or quantitative evaluation of environmental harm associated with the hazard as set out in one or more hypotheses derived from problem formulation. The magnitude of each potential adverse environmental effect should, if possible, be expressed in quantitative rather than qualitative terms (Liu and Agresti 2005), may be used to place identified hazard on a scale of severity.

Step 3: Exposure characterization.

This step is to evaluate the exposure, *i.e.* likelihood of adverse effects occurring, and to estimate the exposure quantitatively. For each hazard identified and characterized, it may not be possible to estimate the exposure (likelihood) precisely. Likelihood of exposure can be expressed either qualitatively using an ordered categorical description (such as "*high*", "*moderate*", "*low*" or "*negligible*") or quantitatively as a relative measure of probability (from zero to one, where zero represents impossibility and one certainty) (EFSA 2010).

Step 4: Risk characterization.

Risk is characterized by combining the magnitude of the consequences of a hazard and the likelihood that the consequences occur (EC, 2002). It is described in this GD as the quantitative or semi quantitative estimate, of the probability of occurrence and severity of harmful effect(s) based on problem formulation, hazard and exposure characterization. The risk characterization should indicate whether the problem formulation (including hazard identification), hazard characterization and exposure characterization are complete.

Step 5: Risk management strategies.

When the risk characterization (step 4) identifies risks, then applicants should propose measures to manage them. These risk management strategies should aim to reduce the identified risks associated with the GM plant to a level of no concern and should consider defined areas of uncertainty.

Applicants should describe the risk management in terms of reducing hazard and/or exposure, and the consequent reduction in risk should be quantified (when possible). Where applicants have identified risk management characteristics (e.g. reduced fertility) in the GM plant which can reduce these risks, then the reliability and efficacy of these characteristics should be assessed. In addition, if applicants place restrictions or conditions on the release of a GM plant in order to reduce risks, then the efficacy and reliability of these measures should be assessed.

Step 6: Overall risk evaluation and conclusions.

An evaluation of the overall risk of the GM plant(s) should be made taking into account the results of the ERA and associated levels of uncertainty, the weight of evidence and the risk management strategies proposed (step 5) in the receiving environment(s). The overall risk

evaluation should result in informed qualitative and, if possible, quantitative guidance to risk managers. The applicants should explain clearly what assumptions have been made during the ERA and what is the nature and magnitude of uncertainties associated with the risk(s). When risks are identified in the overall risk evaluation, applicants should indicate why certain levels of risk might be acceptable.

Conceptual model and analysis plan.

The conceptual model describes a plausible scenario of how harm may arise from use of the GM crop in a way that allows for a characterization of risk. The purpose of the conceptual model is to readily communicate how the environmental risk assessment will be conducted.

Conceptual models take many forms such as simple statements, an outline of activities, flow charts, or diagrams. Conceptual models describe key relationships between the GM plant release and possible environmental consequences of that release. The conceptual model describes the pathway for analysis by setting the problem in perspective and establishing the proposed relationships between exposure and effect (Wolt et al. 2010). Once reasonable scenarios for analysis have been identified and described through conceptual models, they are shaped into an analysis plan. The analysis plan describes the various measures to be used in the assessment, the subsequent characterizations, studies to be conducted, and the appropriate tier for analysis. Importantly, the analysis plan prescribes the manner in which the results should be expressed for risk characterization (Wolt et al. 2010).

Tiered procedure

A tier as a process within risk assessment that is initiated by a decision to collect information and data and ends with a decision either that risk can be and is assessed based on the available information and data, or that the risk cannot be assessed and additional information or data are needed (Andow and Zwahlen 2006). Risk management should be possible at any tier in the assessment process. Tiered testing first addresses broad questions using simple experimental designs with unambiguous outcomes that conservatively cast projections; i.e., appropriately conceptualized early tier assessments should have a low rate of false-negative risk determination but may well have a high rate of false-positives, which will necessitate higher tier assessments (Wolt et al. 2010). The usefulness of tiered testing and assessment for GM plants is being increasingly recognized (Dutton et al. 2003, Garcia-Alonso et al. 2006, Romeis et al. 2008). Published schemes vary in their specifics such as the number of tiers and the nature of tests, but all recognize the critical nature of tiered approaches to iteratively address risk in a manner consistent with the level of concern and the uncertainty in the assessment.

According to Andow and Zwhalen (2006) within a tier, the information and data gathering processes be distinguished from the risk assessment and decision-making processes. This allows the intensiveness of the tiered process to be adjusted depending on the nature

of the risk analysis and the decision to be supported. In addition, when an independent authority (with support from scientific experts) carries out the risk assessment, the assessment can be based on a larger set of data and hence be more accurate than if each single research group assessed the risk based on their data only.
Tiered procedure can drive the Environmental Risk Assessment is an analytical approach with common elements practiced in various regulatory regimes, enhancing the ability of ERAs to answer the appropriate questions necessary for risk management decision making and increase the acceptance of the ERA process globally. While adopting a consistent ERA process may not ensure global acceptance of a particular ERA, it will enhance understanding and increase the ease of evaluation of ERAs conducted within different contexts (Wolt et al. 2010). Despite a general lack of agreement in defining risk analysis, there is a broad consensus that tiering environmental risk assessment is essential to allocate effort to more serious risks while reducing effort on less serious ones (Andow and Zwahlen 2006).

Case by case procedure

According to Snow et al. (2005), the environmental benefits and risks associated with GMOs should be evaluated relative to appropriate base-line scenarios, with due consideration of the ecology of the organism receiving the trait, the trait itself, and the environment(s) into which the organism will be introduced. The results in these early works also implied that risk assessment should be conducted on a case-by-case basis: case-specific risk assessments needed to consider the source and target environments, the biological and ecological characteristics of the transgenic organism, and the scale and frequency of introductions (Andow and Zwahlen 2006).
The case-by-case principle, widely accepted by many international organizations and countries, was applied to ascertain safety assessment procedures and methods for GMOs. According to the case-by-case principle, for each GMO an event-specific safety assessment procedure should be established taking account of its recipient organism, genetic manipulation, target gene, etc. The principle of case-by-case ensured that the safety assessment scheme for a GMO would have enough specificity and sensitivity to find various potential risk factors for edible safety (Deng et al. 2008). For the definition of Risk Assessment method, we follow the case by case procedure, applying the model in different scenarios.
The ERA should be carried out on a case-by-case basis, meaning that the required information may vary depending on the species of GM plants concerned, the introduced genes, their intended use(s) and the potential receiving environment(s), taking into account specific cultivation requirements and the presence of other GM plants in the environment (EFSA 2010).
Furthermore, the results have to be expressed under the worst-case scenario, in the sense that whenever lack of data required formulation of an estimate, that estimate has to be chosen so as not to underestimate negative effects (Perry et al. 2010).

Areas of concerns

EFSA GMO Panel considers seven specific areas of concern to be addressed by applicants and risk assessors during the ERA (1) persistence and invasiveness of the GM plant, or its compatible relatives, including plant-to-plant gene transfer; (2) plant-to-micro-organism gene transfer; (3) interaction of the GM plant with target organisms and (4) interaction of the GM plant with non target organisms, including criteria for selection of appropriate species and relevant functional groups for risk assessment; (5) impact of the specific cultivation, management and harvesting techniques; including consideration of the production systems and the receiving environment(s); (6) effects on biogeochemical processes; and (7) effects on human and animal health.

Each specific area of concern is considered in a structured and systematic way following the six steps described above.

In our study, we focused attention on the first four areas of concern, evaluating the suitability of data collected and comparing them with data reported in literature for the definition of the Risk Assessment method.

Proposed method

The method is focused on the risk assessment step that identifies and evaluates the risks associated with the release and cultivation of GMPs. The proposed method is based on procedures described by de Jesus et al. (2006), following the guidelines described by EFSA (2010). The procedure described by de Jesus et al. (2006), try to assign values for specific parameters; these values make it possible to describe and compare risk measurements with quantifiable tools.

The hazards to be analyzed are organized according to their potential sources of exposure, such as genetic insert, expressed protein, features of the GMP, gene flow, introduction of the technology, and unexpected occurrences (accidents). All concerns related to GMPs, or at least the currently most debated ones, can be arranged in these groups (de Jesus et al. 2006).

Potential Hazards	Criteria for Assessment	Data/Information for Evaluation	Factors of Moderation				Factors of Moderation				
		experimental data from local area or from literature	Damage	Exposure	Prevalent	Index of Risk	Index of Risk (highest value)	Label	Reversibility	Index of Significance	Index of Significance (highest value)
potential source: insert of transgene											
(a) unintended changes in plant characteristics	occurrence of plant diseases										
	growth of plant species										
potential source: interaction of plants with the gene											
(b) persistence and invasiveness	fitness of GM plant										
	GMP (introgressed) wild relatives fitness										
potential source: gene flow											
(c) reducing the genetic diversity of wild population	assess the genetic assimilation										

Fig. 1. Worksheets for the Compilation of the Evidence of Risk. Example of potential hazard of AREA 1 (see the text for details).

The risk assessment methodology is performed in two steps: (1) complete a preformatted worksheet to compile the evidence of risks, and (2) plot the outcome on the Matrix of Assessment. First, a worksheet is constructed to characterize all potential GMP-related hazards and to assign a level of risk and its significance in the context of the activity to be developed.

Fig. 1 shows the worksheet, on this the potential hazards are grouped according to their source of exposure, along with at least one criterion for assessment of each one. These items are predetermined on the worksheet to allow for an accurate evaluation of related risks on the case-by-case basis. In Table 1 each hazard is coded with a letter to identify it in the Matrix of Assessment.

Literature are crucial to support the risk characterization since the assignment of values must correspond with the information described by the user in the corresponding field. The identification of potential hazard is performed according step1 described in EFSA guidelines (see above). An index of risk and a index of significance were computed for each potential hazard using data from literature (Fig. 1). These comprise the "Factors of Moderation," such as damage, exposure, precedent, extent, and reversibility. The index of risk is calculated as the product of: "damage x exposure x precedent"; the damage indicate the level or intensity of the impact that the GMP could have on the system, if the proposed adverse effect actually occurs; the exposure is related to the level that some component is exposed to the damage and precedent considers the previous occurrence of the adverse effect, as a consequence of the event in question (Table1). For additional characterization, the risks must be evaluated according to the context of the activity to be developed. For example, the Index of Significance takes into account the location where the GMP will be cultivated, the identification and evaluation of potential adverse effects, and the evaluation of the current environmental situation. This index is calculated as the product of "extent x reversibility".

The values are shown in Table 2. In this procedure, step 2 and 3 of EFSA guidelines are respected (see above).

Table 1. Values to be attributed to the Factors of Moderation that comprise the Index of Risk.

Index of risk: damage*exposure*precedent		
Factors of moderation	**Levels**	**Values**
Damage	negligible	0
	low	1
	moderate	2
	high	4
Exposure	negligible	0
	low	1
	moderate	2
	high	4
Precedent	no	1
	yes	2

Table 2. Values to be attributed to the Factors of Moderation that comprise the Index of Significance.

Index of Significance: extent*reversibility		
Factors of moderation	**Levels**	**Values**
Extent	Local (contained where GMP is cultivated)	1
	Regional (property or distance of pollination)	2
	Abroad (area affected indirectly)	4
Reversibility	Naturally reversible (without management)	1
	Reversible with simple management (e.g., changing technology)	2
	Reversible with complex management (high costs and use of nonconventional methods)	4
	Irreversible	8

The indexes were combined using a matrix in order to assess the risk for the environment and the measures required to prevent adverse effects of GM plants (Fig. 2).
The "Matrix of Assessment" is constructed with two axes, where the "x" axis stands for the classes of the Index of Risk and the "y" axis stands for the classes of the Index of Significance.
The results from the Index of Risk and the Index of Significance are plotted in the Matrix according to their position (points are plotted using letters that represent each potential hazard). The level of mitigation recommended is classified as:

a. No restrictions - when the hazard does not have a significant chance of being a risk
b. Monitoring required - when the hazard must be observed to avoid adverse effects.
c. Management required - when additional measures must be taken to prevent impacts.
d. Restrictions required - when the activity can be done under restrictive rules or measures and, additionally, frequent observations are required to avoid potential impacts.
e. Not recommended - when hazards show a high level of risk and significance. In this case biosafety measures could be ineffective to prevent or mitigate such risks.

The “Matrix of Assessment” is performed according to step 4 and 5 of EFSA guidelines (2010).

The following step involves compiling and analyzing the results from the matrix and worksheets. Each potential hazard plotted in the matrix requires some measures according to the level of mitigation. These biosafety measures must consider all data described in the worksheet, such as the specificity of the GMP, the activity under analysis, and the environmental situation (step 6 of EFSA guidelines, 2010).

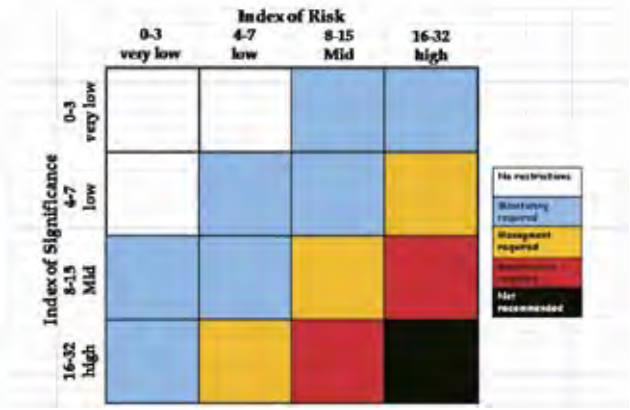

Fig. 2. Matrix of Assessment is the final step of this Risk Assessment tool. The Matrix of Assessment gives an overview of potential hazards and establishes at which level risk management must be taken. The “x” axis represents the classes of the Index of Risk and the “y” axis represents the classes of the Index of Significance.

Case study: Bt-Populus spp.

To better understand the method we propose one case study on possible effects of *Bacillus thuringiensis* (Bt) aspens in the environment, taking into account data reported in literature and the studied area of the DEMETRA project. Poplar trees play an important role in the economic development of forestry (Hu et al. 2001), for this reason one main aim with genetic engineering of trees is to produce plants that are resistant to various types of pests (Genissel et al. 2003, Pena et al. 2001). The most common transformations for pest resistance involve the use of *Bacillus thuringiensis* (Bt) genes, enabling the plant to produce Cry toxins lethal to certain targeted insect pests. *Bacillus thuringiensis* is a soil bacterium with many strains that produce a variety of crystalline protein endotoxins, each of which affects specific groups of insects. Thus, different Bt strains are toxic to lepidopterans (butterflies and moths), coleopterans (beetles), and dipterans (mosquitoes, black flies, and fungus gnats). In these insects, small amounts of the toxin (parts per billion) damage the intestinal system, and the insects typically die within days of a single feeding. One Bt strain is commonly used in both organic and conventional agriculture as an insecticidal spray, and is not toxic to humans or most other organisms, including honeybees and plants (Snow et al. 2005). Genes coding for Bt toxins have been isolated and transformed into the genome of several crop plants, including corn, cotton, and potato and also in forest tree, such as poplar. However, there are considerable risks for the evolution of pest

resistance in wild populations that needs to be evaluated and minimized (Hjalten et al. 2012) The Bt toxin leads to cell damage in the insect mid-gut , more than 150 different Cry proteins have been identified (Schnepf 1998) with examples including Cry3Aa proteins targeting coleopteran insects and the *cry1* and *cry2* families effective against lepidopteran species (Hussein 2005). Previous works reported that in the field trial of insect-resistant transgenic plants, insect larva density, damaged leaf rate and pupa number per unit area in the soil could be good indexes to evaluate insect-resistance of transgenic tree plants. Therefore, it could be beneficial to timber production, environmental protection and insect control if these transgenic trees could be applied. Transgenic poplar could be inter planted with other poplars, or even with other species for insect control as it was done in this field trial (Hu et al. 2001). Certainly, negative effect of these transgenic trees should be further studied.

Hypothetical example (1)

The potential hazards characterized in our case-study were related to four area of interest and were coded with different letters:

1) AREA 1: Persistence and invasiveness of the GM plant or its compatible relatives, including plant-to-plant gene transfer (Fig. 3)

a) unintended changes in plants characteristics
b) persistence and invasiveness
c) reducing the genetic diversity of wild population

AREA 1

Potential Hazards	Criteria for Assessment	Data/Information for Evaluation	Factors of Moderation				Factors of Moderation				
		experimental data from local area or from literature	Damage	Exposure	Persistence	Index of Risk	Index of Risk (highest value)	Extent	Reversibility	Index of Significance	Index of Significance (highest value)
potential source: insert of transgene											
(a) unintended changes in plants characteristics	occurrence of plant diseases	Hilaleon et al., 2012	0	2	1	0	0	2	1	2	2
	growth of plant species	Hilaleon et al., 2012	0	2	1	0		2	1	2	
potential source: increase nur of plants with the genes											
(b) persistence and invasiveness	fitness of GM plant	Davis et al., 2006	1	2	1	2	2	2	2	4	4
	GMP (introgressed) wild relatives fitness										
potential source: gene flow											
(c) reducing the genetic diversity of wild population	assess the genetic assimilation	Ronald, 1992/Lexer et al., 2005 P3 (populus nP. tomentosa)XP11(P. deltoides origin)	2	4	1	8	4	2	4	8	4
	wind direction and natural barriers	Ronald, 1992/Lexer et al., 2005 P3 (populus nP. tomentosa)XP11(P. deltoides origin)	2	4	1	8		2	4	8	
	rate and distance of outcrossing										
	pollen dispersal mediated by natural vectors (wind and animals)	Ronald, 1992/Lexer et al., 2005 P3 (populus nP. tomentosa)XP11(P. deltoides origin)	2	4	1	8		2	4	8	

Fig. 3. Worksheets AREA 1 for the Compilation of the Evidence of Risk for case study: Bt-Populus.

2) AREA 2: Plant to micro-organisms gene transfer (Fig. 4)
   (m) affect rizosphere and soil community
3) AREA 3 Interactions of the GM plant with target organisms (TOs) (Fig. 4)
   (n) ecological processes that affect target organisms biodiversity
4) AREA4: Interactions of the GM plant with non-target organisms (NTOs) (Fig. 4)
   (n2) ecological processes that affect non-target organisms biodiversity.

AREA 2

Potential Hazards	Criteria for Assessment	Data/Information for Evaluation	Factors of Moderation					Factors of Moderation			
		experimental data from local areas or from literature	Damage	Exposure	Precedent	Index of Risk	Index of Risk (highest value)	Extent	Reversibility	Index of Significance	Index of Significance (highest value)
potential source: root exudates of transgenic plants											
(m) affect rizosphere and soil community	persistance in the soil	fungi community/ Danielsen et al., 2012/ Stefani et al., 2009/Hur et al., 2011	1	2	1	2	2	1	2	2	2
	number of cultivable bacteria (human or animal pathogens or non-pathogenic bacteria)	fungi community/ Danielsen et al., 2012/ Stefani et al., 2009/Hur et al., 2011	1	2	1	2		1	2	2	

AREA 3

Potential Hazards	Criteria for Assessment	Data/Information for Evaluation	Factors of Moderation					Factors of Moderation			
		experimental data from local area or from scientific literature	Damage	Exposure	Precedent	Index of Risk	Index of Risk (highest value)	Extent	Reversibility	Index of Significance	Index of Significance (highest value)
potential source: fed with high concentration of transgenic leaf protein											
(n) ecological processes that affect biodiversity	survival and growth rates of TO	Lepidoptera / Hu et al., 2011; Yang et al., 2005 Coleoptera/Li et al., 2011/Su et al., 2011/Cao et al., 2010/Zhang et al., 2011	4	4	1	16	16	1	4	4	4
	mode of action of the active GM plant product towards the TO	Coleoptera/Hallen et al., 2012	4	4	1	16		1	4	4	
	proportion of population of the TO exposed to the GM plant in the receiving environment	Su et al., 2011	4	4	1	16		1	4	4	

AREA 4

Potential Hazards	Criteria for Assessment	Data/Information for Evaluation	Factors of Moderation					Factors of Moderation			
		experimental data from local area or from scientific literature	Damage	Exposure	Precedent	Index of Risk	Index of Risk (highest value)	Extent	Reversibility	Index of Significance	Index of Significance (highest value)
potential source: fed with high concentration of transgenic leaf protein											
(n2) ecological processes that affect biodiversity	survival and growth rates of non target species	Lepidoptera; Hymenoptera; Su et al., 2011/Zhang et al., 2011	1	1	1	1	1	1	1	1	1

Fig. 4. Worksheets AREA 2, 3 and 4 for the Compilation of the Evidence of Risk for case study: Bt-Populus.

The assigned values for the factors of moderation were based on literature data.
Moreover, we observed a breeding event in the studied area (potential hazard c) in AREA1) between two poplars: P3 (*Populus x canescens*) X F1.1(*Populus euramericana*).
This event has to be taken in account because it could be considered a consequence of gene flow and, in case of genetically modified plants the incorporation of transgenes could result in greater invasiveness or loss of biodiversity with related taxa, depending upon the amount of gene flow from generation to generation and the transgenic trait(s), as also reported by Snow et al. (2005).
Considering the distribution of the "letters" inside the Matrix of Assessment (Fig. 5), different risk management could be suggested.
The most part of potential hazards analysed does not pose significant risk, so it does not require additional actions.
Whereas, potential hazard coded as "n" denotes the effect on target organisms and the potential hazard coded as "c" points out the possible consequence of gene flow, they required some restrictions to monitor the risk.

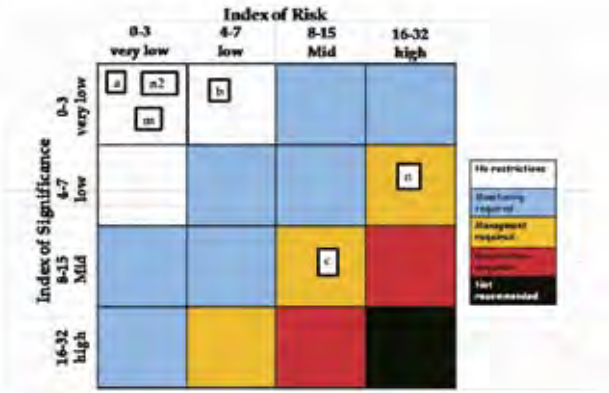

Fig. 5. Matrix of Assessment of case study: Bt-Populus. The Matrix of Assessment gives an overview of potential hazards and establishes at which level risk management must be taken. Letters denotes the potential hazards (see Figs. 3, 4).

Case study: Bt-Brassica napus

A range of crops have been transformed with delta-endotoxin genes from Bt to produce transgenic plants with high levels of resistance to lepidopteran pests.
Parasitoids are important natural enemies of lepidopteran larvae and the effects of Bt plants on these non-target insects have to be investigated to avoid unnecessary disruption of biological control (Shuler et al., 2004).
In particular, transgenic oilseed rape *Brassica napus*, is one of the first genetically modified crops.
Experimental transgenic Bt-producing *B. napus* lines have been generated that confer selective advantage in the presence of Brassica-defoliating insects, including diamond backmoth (*Plutella xylostella* L.), cabbage looper and corn earworm (Halfhill et al., 2002).
A number of transgenic Bt-producing Brassica vegetable crops are also being developed,

using the Bt *cry1Ac* gene designed to control diamondback moth, a pest of global significance in a variety of cruciferous crops (Mason et al., 2003).
Brassica napus is of particular concern, as it is a partially outcrossing species, forms volunteer (feral) populations and has numerous wild relatives.
As a cross-pollinating crop, its natural crossing rate is 30%, and it is liable to cross with other Brassica species.
The ecological risk of transgenic oilseed rape has been concerned by the scientists all over the world.
There are two ways for the pollens flow of transgenic oilseed rape, one could take place between transgenic oilseed rape and other related wild species, and the other could occur between transgenic and non transgenic oilseed rape.
Because the gene can really flow to the conventional oilseed rape, it is necessary to have a sufficient isolation distance in cultivating transgenic oilseed rape (Tang et al., 2005). Therefore the likelihood of pollen-mediated gene flow has been investigated in numerous studies.
Reports of gene flow in *Brassica napus* generally show that the amount of cross-fertilisation decreases as the distance from the pollen source increases.
The occurrence and frequency of pollen-mediated intraspecific gene flow (outcrossing rate) can vary according to cultivar, experimental design, local topography and environmental conditions. The outcrossing rate from one field to another depends also on the size and arrangement of donor and recipient populations and on the ratio between donor and recipient plot size (Hüsken et al., 2007).
The presence of the Bt trait in wild crucifer populations and persistence of the Bt trait in volunteer or feral *B. napus* could also have a potential ecological impact on the insect populations – such as increasing exposure of diamondback moth populations to the Bt toxin and subsequent selection of resistant individuals, or it may even result in lower diamondback moth numbers due to the elimination of an alternate food source (Mason et al., 2003).
Despite the difficulty to compare different experiments with varying levels of outcrossing, we performed a case-study of *Bt- B. napus*.

Hypothetical example (2).

The potential hazards characterized in our case-study were related to three areas of interest and were coded with different letters:

1) AREA 1: Persistence and invasiveness of the GM plant or its compatible relatives, including plant-to-plant gene transfer (Fig. 6)
a) persistence and invasiveness
b) reducing the genetic diversity of wild population

AREA 1

Potential Hazards	Criteria for Assessment	Data/Information for Evaluation	Factors of Moderation				Factors of Moderation				
		experimental data from local area or from literature	Damage	Exposure	Precedent	Index of Risk	Index of Risk (highest value)	Extent	Reversibility	Index of Significance	Index of Significance (highest value)
potential source: increase use of plants with the genes											
(b) persistence and invasiveness	fitness of GM plant	Hendrick et al., 2007	1	2	1	2	2	2	2	4	4
	GMP (introgressed) wild relatives fitness										
potential source: gene flow											
(c) reducing the genetic diversity of wild population	assess the genetic assimilation	*B. napus* x *S. arvensis*	2	4	1	8	8	2	8	8	8
	wind direction and natural barriers	*B. napus* x *S. arvensis*	2	4	1	8		2	4	8	

Fig. 6. Worksheet AREA 1 for the Compilation of the Evidence of Risk for case study: Bt-B. napus.

2) AREA 3 Interactions of the GM plant with target organisms (TOs) (Fig. 7)
(n) ecological processes that affect target organisms biodiversity

3) AREA4: Interactions of the GM plant with non-target organisms (NTOs) (Fig. 7)
(n2) ecological processes that affect non-target organisms biodiversity

AREA 3

Potential Hazards	Criteria for Assessment	Data/Information for Evaluation	Factors of Moderation					Factors of Moderation			
		experimental data from local area or from scientific literature	Damage	Exposure	Precedent	Index of Risk	Index of Risk (highest value)	Extent	Reversibility	Index of Significance	Index of Significance (highest value)
potential source: leaf with high concentration of transgenic leaf protein											
(n) ecological processes that affect biodiversity	survival and growth rates of TO	Legidendrou / Schuler et al., 2004	4	4	1	16	16	1	4	4	4

AREA 4

Potential Hazards	Criteria for Assessment	Data/Information for Evaluation	Factors of Moderation					Factors of Moderation			
		experimental data from local area or from scientific literature	Damage	Exposure	Precedent	Index of Risk	Index of Risk (highest value)	Extent	Reversibility	Index of Signif	Index of Significance (highest value)
potential source: leaf with high concentration of transgenic leaf protein											
(n2)ecological processes that affect biodiversity	survival and growth rates of non target species	Colauttiand Mealey et al., 2001	1	1	1	1	1	1	2	2	2

Fig. 7. Worksheet AREA 3 and 4, for the Compilation of the Evidence of Risk for case study: Bt-B. napus.

The assigned values for the factors of moderation were based on literature data. Moreover, we observed a breeding event in the studied area (potential hazard c) in AREA 1 between *B. napus* and *Sinapis arvensis.* As, in the precedent case-study, considering the distribution of the "letters" inside the Matrix of Assessment (Fig. 8), different risk management

could be suggested. In particular, potential hazard coded as “c” points out the possible consequence of gene flow, it requires some strict restrictions.

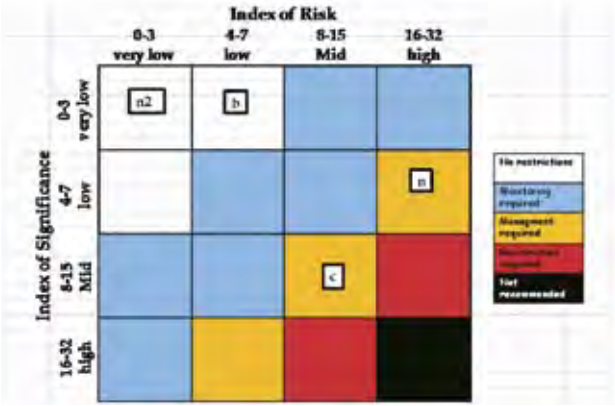

Fig. 8. Matrix of Assessment of case study: Bt-B. napus. The Matrix of Assessment gives an overview of potential hazards and establishes at which level risk management must be taken. Letters denotes the potential hazards (see Figs. 6, 7).

Conclusions

Risk analysis must be undertaken to predict the occurrence of negative impacts on the environment and human and/or animal health. These assessments allow us to define predictive measures to mitigate or avoid the adverse effects that could result from potential or identified hazards (de Jesus et al., 2006). The Matrix assessment for each crop/gene can be used to perform an overall risk evaluation, in this way the overall risk evaluation should result in informed qualitative and, if possible, quantitative guidance to risk managers. At each stage in the model development, we have endeavoured to model ‘worst-case’ scenarios, in which any assumptions would tend towards overestimation rather than underestimation of consequences. This new risk assessment tool will be validated as soon as several users test it with different crops and traits or perform comparative analyses with other methods. Application of ecological expertise and knowledge is essential during all stages of the development of GMOs that are to be released into the environment, from the earliest planning to post- release monitoring and management. The knowledge base about the GM plant, the stability of expression and phenotypic performance of the transgene, and the potential impacts of the GM plant is increasing (Nap et al., 2002). Active involvement of professionals with an understanding of relevant ecological and evolutionary processes can help avert environmental problems (Snow et al., 2005). Moreover, the risks of GMOs are certain or universal. Both may vary spatially and temporally on a case-by-case basis. Comparisons among transgenic, conventional, and other agricultural practices, such as organic farming, will elucidate the relative risks and benefits of adopting GMOs. Measures that prevent transfer of genes that may negatively impact wild populations and that slow the evolution of resistance to the transgenes can minimize some of the possible ecological risks.

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Chapter 5

Geographical information systems for environmental risk assessment and gmo monitoring

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5.1 Introduction

A Geographic Information System (GIS) is a software for the management and analysis of geographic data representing phenomena from the real world in term of spatial position (with respect to a coordinate system) and descriptive attributes (information).
These spatial data and associated attributes can be layered together for mapping and analysis, making GIS a practical tool for environmental monitoring and planning used to assist the decision-making process (Aronoff 1989, Gomarasca 2009). Additionally, together with geostatistics and multivariate statistics, GIS is used by risk assessor to localize environmental risks (Schröder 2006). In the European Union (EU) the environmental risk assessment (ERA) of genetically modified organisms (GMOs) is regulated by EU Directive 2001/18/EC (hereafter referred to as the EU Directive) on the deliberate release into the environment of GMOs (EC 2001). Guidance for assessing potential effects of GMOs on the environment as indicated in the EU Directive are provided by EFSA Panel on Genetically Modified Organisms (EFSA 2009, 2010). A review of the existing regulatory framework and guidance on risk assessment in EU is provided by Aguilera et al. (2013).
The aim of the ERA is to identify potential adverse effects of GMOs on human health and the receiving environment taking into account direct, indirect, immediate and delayed effects of GMO, as well as cumulative long term effects. According to the EU Directive and the EFSA Guidance document, the ERA should be conducted case-by-case on the basis of a step-by-step approach. The following six steps should be considered: i) hazard identification; ii) hazard characterization; iii) exposure assessment; iv) risk characterization; v) application of management strategies for the characterized risks; and vi) determination of the overall risk of the GMO. The EFSA Guidance document identifies seven specific areas of concern to be addressed through the six steps of the ERA. These specific areas

of concern are as follows: i) persistence and invasiveness including plant-to-plant gene flow; ii) plant to micro-organisms gene transfer; iii) interactions of the GM plant (GMP) with target organisms (TOs); iv) interactions of the GM plant with non-target organisms (NTOs); v) impacts on the specific cultivation, management and harvesting techniques; vi) effects on biogeochemical processes; and vii) effects on human and animal health. The EU Directive establishes also that, once a GMO is authorised for marketing, a post-market environmental monitoring must be performed on yearly basis. GMO monitoring has to be appropriate to detect direct and indirect, immediate and long-term as well as unforeseen effects (Züghart et al. 2008). Adverse effects of GMOs on the environment should be investigated taking into consideration biotic and abiotic interactions and their potential impact on several scale, e.g. from field to landscape level, especially when GMOs cultivation on large area is considered and potential environmental effects on the same spatial scale have to be assessed. Indeed small-scale investigations which are currently conducted in laboratories, greenhouses and on single fields might be not suitable to address ecological processes on larger scales (e.g., long distance dispersal of seed and pollen) due to the spatial limitation of the risk assessment. Therefore if in the ERA it is essential to assess potential risks related to small scale releases of GMOs, it is also important to develop and apply appropriate methodologies which allow extrapolation and up-scaling of small-scale processes on larger scales. In this view GIS technology and remote sensing data can provide a concrete contribution to the development of a multi-scale risk assessment of GMOs (Breckling et al. 2011a, b, Reuter et al. 2011). On the basis of our knowledge, the potential of GIS for a large scale risk assessment of GMPs was considered for the first time in the United States, where GIS were used to assess the risk generated by gene flow from GM tree plantations (DiFazio 2002). Gene flow is a key point in risk assessment as it can has adverse environmental effects: if a transgenic crop is released in regions where wild relatives grow, it is expected that spontaneous hybridisation will occur and transgenes may persist and introgress into the natural populations. Gene flow estimates can be integrated with ecological and demographic data in spatially explicit simulation models to allow projections of transgene dispersal over large areas and long time frames. Such models allow the exploration of a large number of cultivation scenarios in order to assist the identification of key parameters controlling gene flow (DiFazio et al. 2004). Since then other studies have been performed to investigate the potential of GIS for ERA and GMO monitoring. For instance, GIS has been used to map the distribution of GMO crops, to model gene flow between genetically modified and conventional field or natural environment due to cross-pollination, and to relate these data with additional environmental data on climate, soil, and agricultural patterns or conservation areas containing protected species and habitats (Craig et al. 2008, Aheto et al. 2011, Breckling et al. 2011, Kleppin et al. 2011, Reuter et al. 2011, Bialozyt 2012). Additionally, GIS has been be used to support sites selection for environmental monitoring, to manage coexistence of GM crops with conventional and organic farming, as well as with nature conservation issues contributing in the determination of the isolation distances needed to assess feasibility of coexistence

measures (Le Bail et al. 2010, Aheto et al. 2011, Kleppin et al. 2011, Moser et al. 2012). GMO monitoring has been also complemented by a Web-based geoinformation system (WebGIS) (Kleppin et al. 2011). A WebGIS is appropriate to build up a data infrastructure for GMO monitoring and data exchange.

It enables access to relevant geodata like basic environmental information, existing monitoring networks related to GMO issues, details on GMO fields and information on protected areas as well as tools for collecting, processing and mapping monitoring results. Implemented GIS tools can also assist the assessment of possible GMO impacts in a spatially discriminated context.

Moreover, a WebGIS could be used to manage the coexistence of GMO with highly sensitive areas (like those within Natura 2000 network where habitat and species conservation is a mandatory goal) detecting possible conflicts during the planning stage. However, the potential of GIS to assist the ERA as indicated in the EU Directive and in the EFSA Guidance document is an aspect to be further developed.

5.2 Objectives

The aim of this Chapter is to describe how GIS-based models might be performed and used by risk assessor to support and complement the ERA of GMPs from local scale to landscape level taking into consideration the EFSA Guidance document (EFSA 2009, 2010). Implications for GMO monitoring are also provided.

Specifically, two gene types (*cry*1ab and *cry*1ac) and two GMPs (poplar an oilseed rape (*Brassica napus*)) were considered. The risk assessment was investigated for four specific areas of concern indicated in the EFSA Guidance document: i) persistence and invasiveness of the GM plant or its compatible relatives; ii) plant to micro-organisms gene transfer; iii) interactions of the GM plant with target organisms (TOs); iv) interactions of the GM plant with non-target organisms (NTOs).

5.3 Materials and methods

5.3.1 Data preparation

The study was carried out in the Migliarino – San Rossore – Massaciuccoli (MSRM) Regional Park (Chapter 1).

Topographic maps and thematic layers were acquired to characterize the environment of the study area and its biodiversity at the landscape level. Topographic maps at the scale of 1:10000 were acquired from Tuscany Region. A land use land cover map (D.R.E.AM 2002) at the scale of 1:15000 was provided by the Regional Park.

A forest type map at the scale of 1:10000 was obtained by polygon delineation of vegetation maps produced by Tomei et al. (2003) and Sani et al. (2010). A map depicting the distribution of agricultural crop types (e.g., maize, sunflower, poplar plantation) at the scale of 1:10000 was provided by the Regional Park. Aerial remote sensing data (year 2007) at the nominal scale of 1:10000 (pixel size = 1 m) were also acquired (Fig. 1). All

these data were projected in a common coordinate system and registered in a geographic database.

Fig. 1. Aerial images (left side) and forest type map (right side) of the study area.

For poplar and oilseed rape data on pollen dispersal mediated by wind were obtained from pollen traps (Chapter 2) and literature data. Information on wind direction and its strength during the blooming season were obtained from weather stations installed within the study area (Chapter 1). For oilseed rape pollen dispersal mediated by animals was also considered using data on insect pollinator registered in the experimental field of sunflower (Chapter 2). Natural and artificial objects (e.g., forest cover, tree plantations, river banks, motorway) that can act as barriers to the pollen dispersal mediated by natural vectors (wind) were identified using the topographic maps, the land use land cover map, and the forest type map. In addition, the distribution of rows of trees within the landscape mosaic was determined by polygon delineation of rows larger than 20 m on the basis of a visual interpretation of aerial images. Data on local biodiversity (plants, animals, and soil micro-organisms) detected in a sample of field plots (Chapter 3) was used to selected TOs and NTOs for gene types *cry*1ab and *cry*1ac. The geographic coordinates of field plots were registered using a Global Positioning System (GPS). The potential distribution of TOs and NTOs within the study area was mapped as follows: GIS tools (overlay and spatial joint) were used to compare field plots distribution with the land use and the forest cover type in order to produce a list of potential habitats for each target and non target species; then, the potential habitats were reviewed by experts in botany, entomology, and soil micro-organisms; finally, the presence of TOs and NTOs detected at the local scale was extended to the landscape level on the basis of potential habitats derived from both the land use land cover map and the forest type map.

5.3.2 GIS-based models

The geographic database was used to develop GIS-based models for ERA of GMPs. To do this a multi-criteria spatial analysis was performed. Transgenes *cry*1ab and *cry*1ac and two GMPs (poplar an oilseed rape) were considered. For poplar four scenarios were investigated on the basis of the following specific areas of concern indicated in the EFSA Guidance document: i) persistence and invasiveness of the GM plant or its compatible relatives; ii) plant to micro-organisms gene transfer; iii) interactions of the GM plant with target TOs; iv) interactions of the GM plant with NTOs. For oilseed rape two scenarios were considered corresponding to specific areas of concern i) and iii). For each specific area, the potential hazards, the criteria for assessment, the index of risk (IoR), the index of significance (IoS), the risk assessment (RA) and the measures required to prevent adverse effects of GM plants indicated by the Environmental Risk Assessment analysis (Chapter 4) were used as reference (Table 1 and Table 2). A sketch of data preparation and GIS modelling can be seen in Fig. 2.

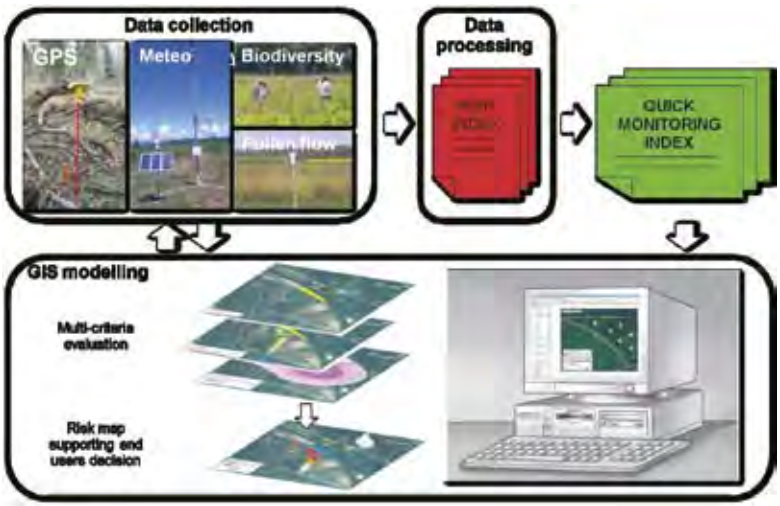

Fig. 2. Overview of GIS analysis for the environmental risk assessment of GMOs.

5.3.2.1 Poplar scenarios

The agricultural crop types map was used to extract the distribution of poplar plantations in the study area. Then we hypothesized that poplar plantations were composed by mature genetically modified poplar trees (*Populus x euramericana*) with gene types *cry*1ab or *cry*1ac. Thus a total of 29 GM poplar plantations covering a total area of 291 hectares were considered for GIS modelling (Fig. 3a). In poplar scenario 1 the gene flow between GM trees and poplar trees in the surrounding ecosystems was investigated as potential source for exposure (EFSA specific area 1), considering that the breeding between GM trees and non GM trees represents a potential hazard reducing the genetic diversity of wild population. Pollen and seed dispersal mediated by wind, wind direction and natural barriers

were used as criteria for assessment. In the MSRM Regional Park naturally originated poplar trees (*Populus alba* L. and *Populus x canescens* ((Aiton) Sm.)) can be found in mixed broadleaved forests dominated by hygrophilous species and in the wetlands of Massaciuccoli lake (Chapter 3). Therefore the potential distribution of poplar trees in the study area was assumed to be equal to the distribution of hygrophilous forests, meso-hygrophilous forests, and wetlands (Fig. 3a). Gene flow due to pollen flow was modeled taking into consideration data from pollen traps and literature data. Data from pollen traps showed that a considerable presence of pollen can be found up to a distance of 540 m (Section 2.1). However, because no traps where positioned at a distance greater than 540 m, we considered that, when barriers do not exist, poplar pollen can be dispersed by wind up to a distance of 2 km (Imbert and Lefèvre 2003, Slavov et al. 2010) as indicated by genetic data in the Massaciuccoli lake (Section 2.2). For poplar scenarios forest cover and rows of trees were used as natural barriers; artificial barriers were not considered. Data from weather stations showed that in the study area wind direction changed during the blooming season. Accordingly we did not consider wind direction on the basis of a precautionary principle. Therefore pollen dispersal was modeled using a buffer 2 km large delineated around the edges of GM poplar plantations, then the buffer was clipped to take into consideration the presence of natural barriers (Fig. 3b). In addition, we considered that pollen can penetrate forest cover up to a distance of 50 m (Imbert and Lefèvre 2003, Slavov et al. 2010) as indicated by genetic data in Fortino Nuovo locality (Section 2.2). Pollen dispersal and the distribution of wild relatives were intersected to map the potential areas where breeding between GM trees and poplar trees in the surrounding environments might occur. Additionally, to assess the persistence and invasiveness of GM poplar in the ecosystem and the risk of breeding for the progeny, we considered that, if barriers do not exist, poplar can disseminate up to a distance of 2 km, while, inside the forest, we considered that poplar dissemination occur within 50 m. Finally, the environmental risk for poplar scenario 1 was assessed taking into consideration the IoR, the IoS, and RA established by the ERA analysis (Table 1). In poplar scenario 2 the environmental risk due root exudates released by transgenic poplar trees was investigated as potential source for exposure (EFSA specific area 2), considering as potential hazard that root exudates might affect the rhizosphere and soil community. To map such a risk, which is a consequence of risk investigated in poplar scenario 1, we supposed that gene transfer from GM poplar trees to soil micro-organisms (Fig. 3c) occur in those areas where a risk of breeding (including the risk for the progeny) between GM poplar trees and its wild relatives exist.
As for poplar scenario 1, the environmental risk was assessed taking into consideration the IoR, the IoS, and RA indicated by the ERA analysis (Table 1). In poplar scenario 3 the interactions of the GM poplar trees with TOs were addressed (EFSA specific area 3). In this case we considered as potential hazard that GM plants affect biodiversity reducing the survival and growth rates of TOs, as TOs feed with high concentration of transgenic leaf protein. The proportion of population of TOs exposed to transgenic plants in the receiving environment was the criteria for assessment. To do this we investigated the distribution

of TOs exposed to GM poplar trees. In the MSRM Regional Park the larvae of Diptera Tabanidae and the lepidopteran Noctuidae were detected during the fieldworks (Section 3.2). Because poplar plantations can be damaged by these two insects, Tabanidae larvae and Noctuidae were considered as TOs. The distribution of TOs in the study are was mapped taking into consideration that Diptera Tabanidae and lepidopteran Noctuidae can be found in hygrophilous and meso-hygrophilous forests (Fig. 3d). Then the distribution of TOs exposed to GM poplar trees was obtained by means of an intersection between the distribution of TOs and the distribution of potential areas for breeding detected in poplar scenario 1. Once again the environmental risk was assessed taking into consideration the IoR, the IoS, and RA provided by the ERA analysis (Table 1).

Table 1. EFSA specific areas of concern, potential hazards, criteria for assessment, information for evaluation, index of risk (IoR), index of significance (IoS), risk assessment (RA) and measures required to prevent adverse effects for poplar scenarios (From Chapter 4, modified to adapt risk assessment to GIS modelling).

Poplar scenario	EFSA specific area of concern	Potential source of exposure and potential hazard	Criteria for assessment	Data/information for evaluation	IoR	IoS	RA
1	1	Gene flow (c) Reduction of the genetic diversity of wild population	Natural barriers Wind direction Pollen dispersal mediated by natural vectors (wind) Seed dispersal mediated by natural vectors (wind) Distribution of wild population	Topographic map, forest map, remote sensing data Weather stations Data from pollen traps and literature data (max 2 km without barriers; max 50 m inside the forest) Literature data (max 2 km without barriers; max 50 m inside the forest) Potential distribution from existing maps	8	8	Management required
2	2	Root exudates of transgenic plants (m) Alteration in the rhizosphere and in soil community	Persistence in the soil Proportion of population of micro-organisms exposed to transgenic plants in the receiving environment	Literature data Potential distribution of transgenic plants from poplar scenario 1 Potential distribution of micro-organisms from existing maps	2	2	No restrictions
3	3	TOs feed with high concentration of transgenic leaf protein (n) Ecological processes that affect biodiversity	Proportion of population of TOs exposed to transgenic plants in the receiving environment	Potential distribution of transgenic plants from poplar scenario 1 Potential distribution of TOs from existing maps	16	4	Management required
4	4	NTOs feed with high concentration of transgenic leaf protein (n2) Ecological processes that affect biodiversity	Proportion of population of NTOs exposed to transgenic plants in the receiving environment	Potential distribution of transgenic plants from poplar scenario 1 Potential distribution of NTOs from existing maps	1	2	No restrictions

In poplar scenario 4 the interactions of the GM poplar trees with NTOs were investigated (EFSA specific area 4). In this scenario we considered as potential hazard that GM poplar trees affect biodiversity reducing the survival and growth rates of NTOs. The distribution of NTOs exposed to GM poplar trees was used to assess the proportion of population exposed to transgenic plants. Specifically, micro-arthropods in the soil like Acarina, Collembola and Formicidae were taken into consideration as NTOs because these species can feed with vegetable material (e.g. leaves) released by GMPs, or with vegetable material covered by GM pollen granules. The distribution of NTOs in the study area was mapped taking into consideration that micro-arthropods prevail in forest soils dominated by mixed broadleaved species (Fig. 3e). Then the distribution of NTOs exposed to GM poplar trees was obtained using the method described for poplar scenario 3. Finally, the environmental risk for poplar scenario 4 was assessed using the IoR, the IoS, and RA established by the ERA analysis (Table 1).

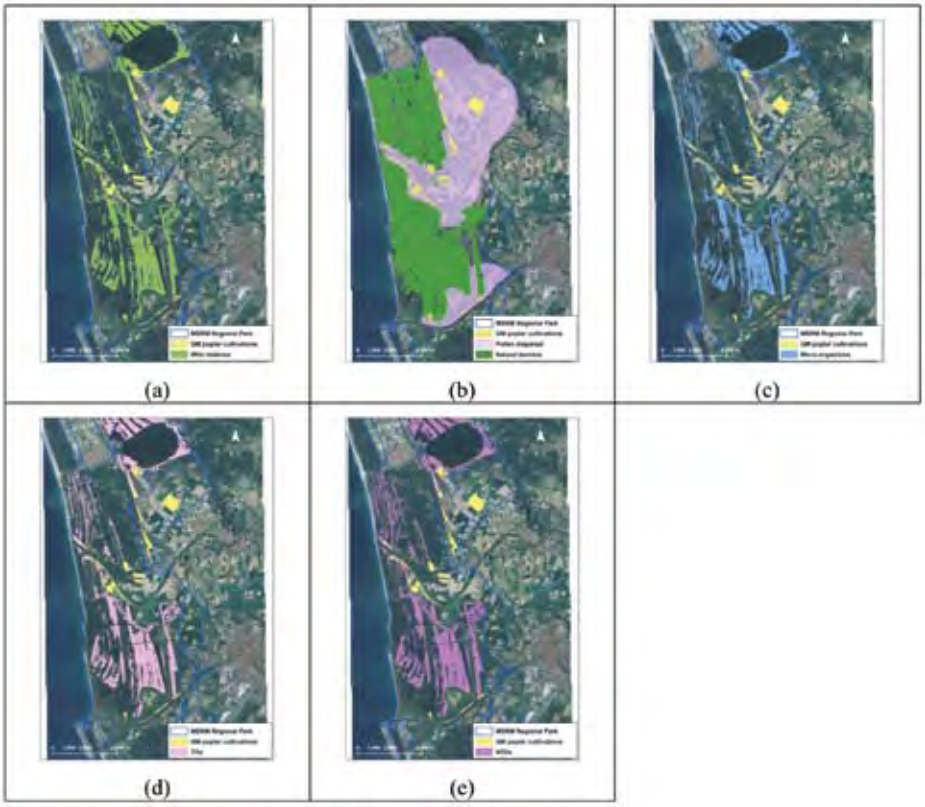

Fig. 3. Poplar scenarios: a) distribution of GM poplar cultivations (Populus x euramericana) and of its wild relatives (Populus alba L. and Populus x canescens ((Aiton) Sm.)); b) distribution of pollen dispersal and of natural barriers; c) distribution of micro-organisms; d) distribution of target organisms (TOs) (larvae of Diptera Tabanidae and lepidopteran Noctuidae); e) distribution of non target organisms (NTOs) (micro-arthropods in the soil).

5.3.2.2 Oilseed rape scenarios

The agricultural crop types map was used to extract the distribution of oilseed rape cultivations in the study area, resulting in 13 oilseed rape cultivations covering a total area of 179.8 hectares. Then we hypothesized that genetically modified oilseed rape with gene types *cry*1ab or *cry*1ac were cropped in the MSRM Regional Park (Fig. 4a). For oilseed rape two scenarios were considered (Table 2).

In oilseed rape scenario 1 the persistence and invasiveness of the GM plant or its compatible relatives was investigated (EFSA specific area 1), considering as potential source for hazard that gene flow might reduce the genetic diversity of wild population. Pollen dispersal mediated by natural vectors (wind and animals), and natural and artificial barriers were used as criteria for assessment. As for poplar scenarios, wind direction was not considered as it changed during the blooming season. Data from field plots (Section 3.1) indicated that *Sinapis arvensis* (Brassicaceae family) is the wild relatives for oilseed rape to be considered in the studied area, and that *S. arvensis* can be found in the environment of fallow lands surroundings the cultivated areas. Therefore the distribution of *S. arvensis* was assumed to be equal to the distribution of non cultivated lands obtained from vegetation and agricultural crop type maps (Fig. 4a). On the basis of field observations we considered also the presence of *S. arvensis* within a buffer 5 m large delineated around the edges of oilseed rape cultivations. Being oilseed rape characterized by anemophilous and entomophilous pollination, pollen dispersal was modeled using data from pollen traps and data on insect pollinator (*Apis mellifera*) registered in the study area (Section 2.1). Data from pollen traps recorded in 2011 were used to build a mathematical function indicating the number of pollen granules / cm^2 in relation to the distance from cropped areas. The function was as follow (r = 0.82) and it was used to asses the maximum distance reached by pollen:

$$y = 247.4\, x^{-0.54}$$

where, y is the number of pollen granules / cm^2, and x is the distance in meters from oilseed rape cultivation. For insect pollinator a maximum distance of 1 km was considered. For oilseed rape scenarios, forest cover (including tree plantations) and rows of trees were considered as natural barriers, while river banks and motorway as artificial barriers. These barriers were used to clip the map of pollen dispersal mediated by natural vectors (Fig. 4b), then the resulted layer was intersected with the potential distribution of *S. arvensis* to map the potential areas where breeding between GM oilseed rape and its wild relative *S. arvensis* might occur. Finally, the environmental risk was assessed using the IoR, the IoS, and RA established by the ERA analysis (Table 2).

In oilseed rape scenario 2 the interactions of the GM plants with TOs were addressed (EFSA specific area 3). To do this Lepidoptera was identified as TO on the basis of biodiversity data collected in the plots (Section 3.2). The distribution of TO was assumed to be equal to the distribution of its habitats: hygrophilous and meso-hygrophilous forests, and sowable

lands (Fig. 4c). Then the distribution of TO was intersected with potential areas where breeding between GM cultivations and *S. arvensis* might occur. Finally, the environmental risk was assessed using the IoR, the IoS, and RA indicated by the ERA analysis (Table 2).

Table 2. EFSA specific areas of concern, potential hazards, criteria for assessment, information for evaluation, index of risk (IoR), index of significance (IoS), risk assessment (RA) and measures required to prevent adverse effects for oilseed rape scenarios (From Chapter 4, modified to adapt risk assessment to GIS modelling).

Oilseed rape scenario	EFSA specific area of concern	Potential source of exposure and potential hazard	Criteria for assessment	Data/information for evaluation	IoR	IoS	RA
1	1	Gene flow (c) Reduction of the genetic diversity of wild population	Natural and artificial barriers Wind direction Pollen dispersal mediated by natural vectors (wind and animals) Distribution of wild population	Topographic map, forest map, remote sensing data Weather stations Data from pollen traps; data on insect pollinator registered in the study area Potential distribution from existing maps	8	8	Management required
2	3	TOs feed with high concentration of transgenic leaf protein (a) Ecological processes that affect biodiversity	Proportion of population of TOs exposed to transgenic plants in the receiving environment	Potential distribution of transgenic plants from oilseed rape scenario 1 Potential distribution of TOs from existing maps	16	4	Management required

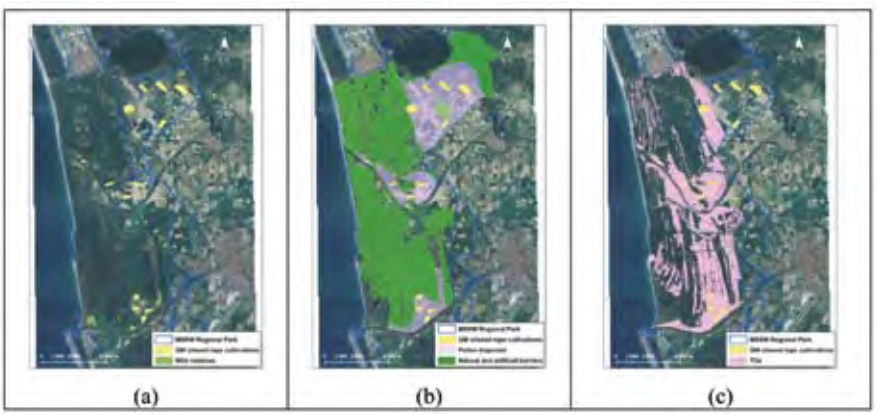

Fig. 4. Oilseed rape scenarios: a) distribution of GM oilseed rape cultivations and of its wild relatives (Sinapis arvensis); b) distribution of pollen dispersal and of natural and artificial barriers; c) distribution of target organisms (TOs) (Lepidoptera).

5.4 Results

5.4.1 ERA for poplar scenarios

In poplar scenario 1 the potential hazard due to breeding between GM poplar cultivations and its wild relatives in the surrounding environments was investigated. Based on the information presented in Table 1, and using the spatial dataset available for the study area, it seems that GM pollen responsible of gene flow could affect important habitats hosting naturally originated poplar population. The assessment of this potential hazard indicates that some management measures are required to mitigate the risk. The results of the ERA performed with GIS modelling indicate that the area of the habitats exposed to risk of breeding is of 187.9 hectares. The area exposed to risk enlarge up to 822.5 hectares when the risk for the progeny is considered, representing 23.5% of the total area of the habitats (Table 3). For forestry habitats the risk concentrates along the edges of forest cover because tree crowns acts as barriers against pollen dispersal. Instead, in the wetlands of Massaciuccoli lake, the risk is larger because natural barriers are scarce (Fig. 5a). In poplar scenario 2 the potential hazard for soil micro-organisms due to root exudates released by transgenic poplar trees was considered. The results of the spatial analysis indicated that the areas exposed to this potential hazard correspond to the areas exposed to the hazard investigated in poplar scenario 1 (Table 3). However, using the evaluation adopted by the ERA analysis, it seems that this issue does not pose significant risk in the study area, so it does not require additional actions (Fig. 5b). It is worth noting that GIS modelling was extended in the area of Massaciuccoli lake where data on soil micro-organisms where not sampled. In case of scenario 3 the interactions of the GM poplar cultivations with TOs (larvae of Diptera Tabanidae and lepidopteran Noctuidae) were examined. The level of mitigation of this issue requires some management measure. These would be developed together with the measures required to mitigate the risk of breeding considering that these two potential hazards have the same distribution (Table 3 and Fig. 5c).

In poplar scenario 4 the interactions of the GM poplar cultivations with NTOs (micro-arthropods in the soil) were addressed. The spatial distribution and the area of this potential hazard can be seen in Figure 5d and in Table 3, respectively. The assessment of this issue for the study area indicate that no restrictions are required to mitigate the potential adverse effects of GM poplar cultivations on NTOs.

Table 3. Poplar scenarios: extent of the area exposed to risk. The extent of the area (in %) of wild relatives (scenario 1), micro-organisms (scenario 2), TOs (scenario 3), and NTOs (scenario 4) exposed to risk respect to their total area is indicated in brackets.

Poplar scenario	EFSA specific area of concern	Area of GM poplar cultivations	Area exposed to risk
		ha	ha (%)
1	1	291	822.5 (23.5)
2	2	291	822.5 (23.5)
3	3	291	822.5 (23.5)
4	4	291	822.5 (23.5)

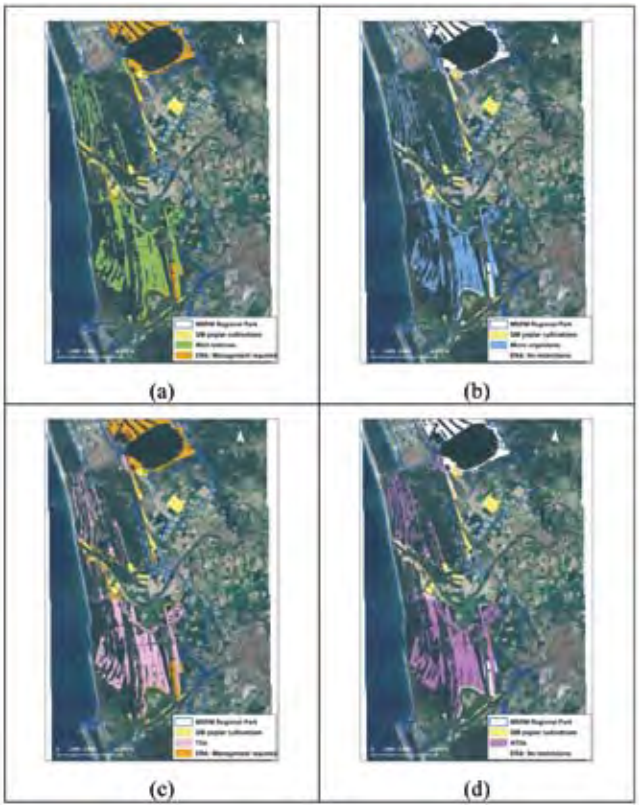

Fig. 5. ERA and measures required to prevent adverse effects of GM poplar cultivations (Populus x euramericana) in the study area: a) risk for breeding between GM poplar and its wild relatives (Populus alba L. and Populus x canescens ((Aiton) Sm.)) including their progeny; b) risk for micro-organisms in the soil; c) risk for target organisms (TOs) (larvae of Diptera Tabanidae and lepidopteran Noctuidae); d) risk for non target organisms (NTOs) (micro-arthropods in the soil).

5.4.2 ERA for oilseed rape scenarios

For oilseed rape two scenarios were studied. The potential hazard that gene flow from GM oilseed rape cultivations might reduce the genetic diversity of *S. arvensis* was investigated in the scenario 1. Based on ERA analysis this issue requires some management measures to mitigate the risk. The ERA assisted by GIS analysis shows that GM pollen could be dispersed by natural vectors (wind and insects) on most of non cultivated lands where *S. arvensis* grows (Fig. 6a). Overall, the area exposed to risk of breeding is of 58.7 hectares, corresponding to 64.1% of the total area occupied by *S. arvensis* (Table 4). It is worth noting that the lack (or the scarce presence) of natural and artificial barriers in agricultural ecosystems within the studied area, expose conventional cultivations and wild plants to a large risk of breeding.

In oilseed rape scenario 2 the potential hazard due to the interactions of the GM *B. napus* cultivations with TOs (*Lepidoptera*) were considered. The level of mitigation of this issue determined by the ERA analysis was “management required”. In the study area

Lepidoptera can be found in several habitats and its potential distribution cover a area of about 3391 hectares. The results provided by the spatial analysis show that the potential hazard affect almost 33% of the potential distribution area of TOs (Table 4), comprising forest and agricultural ecosystems (Fig. 6b).

Table 4. Oilseed rape scenarios: extent of the area exposed to risk. The extent of the area (in %) of wild relatives (scenario 1) and TOs (scenario 2) exposed to risk respect to their total area is indicated in brackets.

Oilseed rape scenario	EFSA specific area of concern	Area of GM oilseed rape cultivations	Area exposed to risk
		ha	ha (%)
1	1	179.8	58.7 (64.1)
2	3	179.8	1120.9 (33.1)

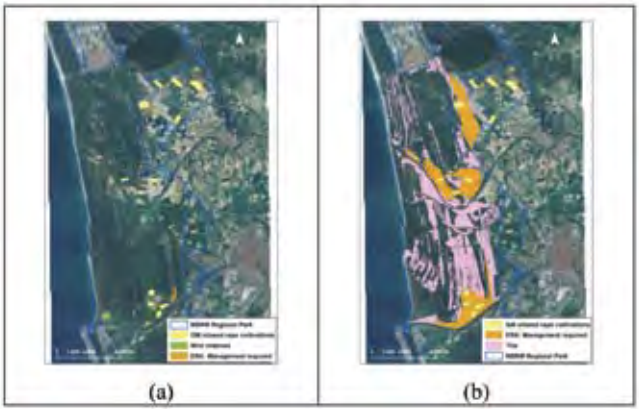

Fig. 6. ERA and measures required to prevent adverse effects of GM oilseed rape cultivations in the study area: a) risk of breeding between GM oilseed rape and its wild relatives (Sinapis arvensis); b) risk for target organisms (TOs) (Lepidoptera).

5.5 Conclusions

Risk assessment is necessary to prevent the occurrence of adverse effects of GMOs on human and animal health and on the receiving environment. In the European Union the ERA of GMOs is regulated by EU Directive 2001/18/EC.

In this Chapter we explored the potential of GIS technology to assist and complement the ERA of GMPs for four specific areas of concern indicated in the EFSA Guidance document. Potential hazards, criteria for assessment, risk assessment and measures needed to mitigate the adverse effects of GMPs defined by the ERA method were used as reference for GIS modelling.

Our results show that GIS and geographic data can assist risk assessor from the description of the characteristics of the receiving environments where GMP is likely to be distributed to the risk analysis in a spatially discriminated context.

Specifically, GIS was used to collect existing maps for the entire study area to characterize

the environments in term of land use, agricultural crop types, and habitats. These geographic data were used also to extent biodiversity data collected in a sample of plots from the local scale to the landscape level.
Field data, literature data and spatial analysis were of particular importance to model both pollen and seed dispersal in order to predict the occurrence of negative impacts on wild relatives as well as on target and non target organisms. Our results indicate that natural and artificial barriers are important to mitigate the risk of breeding between GMP and its compatible relatives. Indeed, in the study area this potential hazard was large in the agricultural ecosystems and in the wetlands of Massaciuccoli lake because of lack of barriers. This outcome should be taken into consideration as mitigation measure, suggesting, for instance, the importance of conserving rows of trees (e.g., windbreaks) and shrubs along the rivers and in agricultural landscapes.
In risk assessment the risk evaluation should result in informed qualitative and, if possible, quantitative guidance to risk managers. The method used to develop the ERA with GIS-based models is based on a multi-criteria spatial analysis. Information produced by GIS (maps and statistics) for each potential hazard considered by the ERA method for four specific areas of concern indicated in the EFSA Guidance document provides qualitative and quantitative information, indicating the spatial distribution of the environments exposed to risk, the measures required to mitigate the risk, and the extent of the area exposed to risk. These information are useful also for GMOs monitoring, for instance to select checkpoints and their distribution.
In conclusion, the results of the DEMETRA project are important to further develop existing guidance on the environmental risk assessment and monitoring of GM plants according to the EU Directive 2001/18/EC on the deliberate release into the environment of genetically modified organisms. In this view, Geographical Information Systems represent a useful tool available for risk assessor and risk manager.

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Chapter 6

Participation of the region of tuscany in demetra project

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1. European Community main news on GMOs

On July 2010, the European Community (EC) regulatory scenario underwent a significant turn with the emanation of a proposal to amend the Directive 2001/18/EC. This proposal represented a big opportunity for the Member States to declare themselves GMOs free, providing them with the possibility of restricting or banning GMO crops on their territories. It also added motivations different from the ones strictly connected with environmental and health risks, as socio-economic reasons. On July 5th 2011, The European Parliament adopted a legislative resolution, followed by a long phase of unsuccessful discussions, the only result being the provision's standstill. In the light of this, on January 2013 EC decided to suspend the authorisation procedure for new type of GMO cultivations, until a final decision will be taken by the EC Board on the proposal promoted by the executive committee to regulate GMO farming in Europe, and in any case until the end of its mandate in 2014. During the last 14 years, only two authorisations have been granted all over Europe: Amflora potato, not for feed uses, and Mon 810 corn, with a global area occupied by GMO planting below 100,000 hectares. So far, only Spain, Portugal and few Eastern Countries, with Poland among them, grow GMO corn on a large scale. The global situation is far different, with crops spread over an area of more than 160 million hectares, the United States being at the forefront.

A new chapter on the GMOs issue opened up, which saw the Member States heading in a random fashion. Eight Countries (France, Luxembourg, Austria, Germany, Poland, Bulgaria, Greece and Hungary) opted for the safeguard clause, provided for by art.23 of Dir. 2001/18 on intentional GMOs emission in the environment. This clause allows GMO farming ban on their territory, upon submission to the EC of a file integrated with scientific data proving an existing harm for health or the environment.

2. The National situation

Italian regions have always expressed their firm opposition to authorizing GMO crops

on their territory; in 2010, they also refused the coexistence measurements, asking the Government, like other European Countries, the safeguard clause activation. So far, this is the only feasible way of reaching the final goal of keeping the territory GMOs free. The Government didn't agree to this request for years, leaving the Italian situation hung in the balance without neither coexistence measurements, nor the safeguard clause. Nonetheless, D.lgs. 212/01 "*Implementation of the Directive 98/95/EC regarding seeds mass production, the communal catalogue of farming plants' variety and related inspections*" allowed to maintain the territory GMOs free up to now. Article 1 clause 2 of the decree states that seeds farming must be subject to the authorisation from the Agriculture Department in concert with environment and health. This will guarantee avoiding contact between GMOs and traditional seeds farming, and will also prevent biologic harm.
Nevertheless, on September 2012 a judgement was passed by the Court of Justice, ratifying that an EC Member State cannot halt GMO farming while waiting for Regions to adopt a regulation allowing the coexistence between traditional and genetically modified crops. Moreover, a Member State cannot promote interior authorisation processes. With this judgement the moratorium de facto considered by our legislation failed. This open two possible routes, doable at the same time: start the safeguard clause activation and resume the work on coexistence guidelines (with a final goal to have very restrictive rules, practically preventing GMO crops).

3. Region of Tuscany and GMOs

As the majority of all the Italian regions, Region of Tuscany has always been contrary to the presence of GMOs on its territory. GMO farming in Tuscany would contradict the development strategy of agricultural holdings and of the entire agricultural and food system, based on quality, uniqueness, link with the territory and differentiation regarding homologated goods already on the market.
This would result into a damage for the Region of Tuscany image, based on territorial, cultural and artistic resources which uniquely blend into the international outlook. Therefore, there is no need for GMO crops, which do not represent an opportunity for Tuscan farmers, clashing instead with their productive vocation.
This position is supported not only by the opposition of the majority of citizens and farmers to GMOs, but also by the undeniable economic unsustainability of the coexistence between GMO and not-GMO farming, due to the socio-economic features of Tuscan farms and the geomorphologic peculiarities of the territory.
For this reason, Region of Tuscany adopted in 2000 the L.R. 53 " Regional protocol in terms of GMOs", which recognises the precaution and the safeguard of health and environment through:

- Prohibition of growing and producing GMO species in Tuscany;
- Obligation to specify on the label the possible presence of GMOs in foodstuffs put on the market in Tuscany.

Inspections are expected to verify the observance of this regional regulation. The Regional Phytosanitary Service carries out inspections on crops and imported seeds, while controls on foodstuffs are the responsibility of the General Direction for citizenry rights and social cohesion, Prevention Service in public and veterinary healthcare, through the Local Health Authorities.
Many other Italian regions, such as Lazio and Friuli Venezia Giulia, issued regional bylaws during the years, banning GMOs farming on their territories. Unfortunately, these bylaws have never been notified to the EC, like so the L.R. 53/00. Due to this and to the following normative developments, our regional regulation is today at risk of legitimacy.

4. Region of Tuscany and Life + DEMETRA Project

The Life + Demetra Project places itself right in the heart of this legislative uncertainty. Region of Tuscany endorsed project Demetra in 2008, to answer the needs for protection of the territory from potential transgenic plantations and to support the growing debate with scientifically proven data.

The Quick Monitor Index (QMI) is capable of predicting the potential impact of transgenic farming on ecosystems, thus providing crucial information to define the operating methods for environmental monitoring. This is a very innovative procedure of environmental risk assessment for transgenic plantations, which turned out to be very useful for Region of Tuscany to protect its territory. Furthermore, QMI, conveniently adjusted to the different geographic situations, might be used in the future as a forecasting model to support with unquestionable data the possible ban of GMO farming in a specific area.

Note

Note

Stampa: Tipografia Francesconi, Lucca