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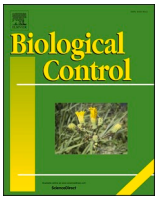

# Disentangling omnivory of heteropteran and coccinellid predators present in peach and alfalfa crops by metabarcoding analysis

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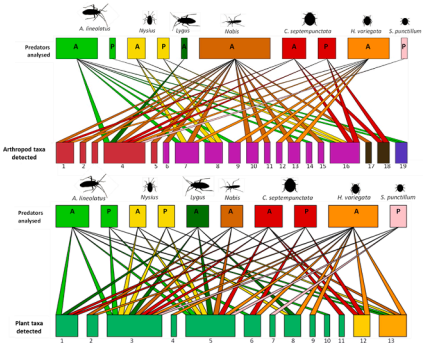
## HIGHLIGHTS

- The approach used allows disentangling the omnivory of insects known as phytophagous.
- This information sheds light about their role as potential biological control agents.
- Revealed trophic interactions also show insect movement among crops and other plants.
- This could indicate the role of those plants in attracting predators in the studied crops.

## GRAPHICAL ABSTRACT




## ARTICLE INFO

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## ABSTRACT

Ecosystems management is essential for the biological control of arthropod pests in agriculture. For this, it is necessary to know which arthropod and plant resources are the most used by the generalist predators present in the studied agroecosystem. Molecular approaches, like high-throughput sequencing (HTS) are nowadays a key tool to disentangle the resources consumed by each predator species. In this study we use a multi-primer metabarcoding approach with pooled samples to screen the most common trophic interactions of four heteropteran and four coccinellid species. They were the most common when they were collected in a peach and in an adjacent alfalfa crop at different dates in two consecutive years. The HTS analysis of 433 heteropteran and coccinellid predators showed that they ingested 27 arthropod taxa, including a potential pest of peach not cited until now, and 14 plant taxa. Detection of some ingested arthropod taxa and plant DNA showed that those predator species foraged on non-crop plants, which play a role in attracting or maintaining these predators close or in the crops. This metabarcoding approach also showed the omnivory of those heteropteran and coccinellid species, important information to improve biological control programs.

## 1. Introduction

Nowadays, the studies related with ecosystem services in agroecosystems are focused on understanding the functional features provided by insects, like biological control (Perović et al., 2018; Demestihas et al., 2017). It is estimated that 20 to 40 % of the global

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crop production is annually lost by the action of insect pests (FAO, 2020), increasing in recent years due to the climate warming that alters relevant biological insect features (Deutsch et al., 2018). It is known that the efficiency of biological control depends on the food web of the agroecosystem. In this context the role of each species could become fundamental to manage species or functional groups (Bohan et al., 2013). Despite the numerous studies in insect food webs, some factors that influence them are still not well known, like predator diversity and its importance in the ecosystem management, or how the role of omnivorous insects affects other organisms in an agroecosystem (Schoenly et al., 1991; Lancaster et al., 2005; Krimmel, 2011). Insect omnivory is common and widespread across agricultural ecosystems (Eubanks et al., 2003), and to know which plant and prey resources are consumed allows developing efficient pest control programs (Krimmel, 2011). Even if the study of food webs is complicated and often requires years of ecological observations and considerable taxonomic expertise (Clare et al., 2019), nowadays some approaches like the analysis of fatty acids, the use of stable isotopes, or DNA molecular approaches are of great help (Nielsen et al., 2018). High throughput sequencing (HTS) has emerged as a suitable option for dietary studies of environmental samples (González-Chang et al., 2016; Roslin & Majaneva, 2016; Nielsen et al., 2018; Batuecas et al., 2021; 2022), showing some advantages with respect to other methods, like a higher taxonomic resolution, the detection of unexpected trophic interactions or the correct identification of species that are not easy to identify (Galan et al., 2018; Taberlet et al., 2018, Nichols et al., 2018). The use of plant and arthropod primers to study omnivory and the analysis of a higher number of samples in sample-pools, saving time and cost, have been reported when using this method (Batuecas et al., 2021; 2022).

In the study of a food web within an agroecosystem, both the landscape configuration and composition must be considered, because they influence the abundance of insects (pests and predators) and the insect community structure (Marino & Landis, 1996; Bianchi et al., 2006; Rusch et al., 2010; Tscharntke et al., 2012), particularly considering the local variables of the area of study (Clemente-Orta et al., 2020, Madeira et al., 2022) and the insect's movement between crops (Madeira et al., 2014; 2019). The influence of the landscape is especially relevant in the Ebro Basin (NE Iberian Peninsula), composed by a mosaic of crops dominated by arable crops and alfalfa, scattered with fruit orchards and natural or semi-natural habitats (Clemente-Orta et al., 2020; Madeira et al., 2022). In this area, alfalfa fields host major insect predators, like coccinellids and heteropterans, which also play an essential role in the conservation biological control (CBC) of adjacent crops, like maize (Pons et al., 2005, 2009; di Lascio et al., 2016; Madeira et al., 2014; 2019). Even if peach orchards in the same area of study have a growing economic importance, their role in CBC has been much less studied than alfalfa crops. Some studies show the presence of anthocorids, syrphids and hymenopteran parasitoids as biocontrol agents (Avilla et al., 2009; Aparicio et al., 2019; 2020; 2021), but their role is still not well known.

Heteropterans are the most abundant arthropod predators in the Ebro Basin (Pons et al., 2005). It is known that the species of this Suborder belong to two main lineages with different feeding habits according to their adaptative evolutions, either omnivorous or strict herbivorous (Eubanks et al., 2003). In this area, some heteropterans, like nabids and mirids have been traditionally considered as predators of aphids, as well as other prey species, but information about their potential plant consumption is unknown (Pons et al., 2009). On the other hand, mirids and lygaeids have been considered as phytophagous in other regions, even as pests of alfalfa or peach (Del Rivero & García-Marí, 1983; Schaber & Entz, 1994; Blando & Mineo, 2005). Coccinellids have been traditionally considered as true predators because most of the species of this family have been shown to feed on aphids, although they could also diversify their diet with additional food including other invertebrates, and even pollen, nectar, and spores (Giorgi et al., 2009; Escalona et al., 2017). Nevertheless, in the Ebro Basin, they have been considered as aphid predators exclusively (Pons et al., 2009).

In the present study, we use an HTS multi-primer metabarcoding approach to analyse four heteropteran and four coccinellid taxa to detect their most common trophic interactions regarding arthropod, as well as plant resources. They were collected in an alfalfa and in a peach crop in the Ebro basin, both adjacent and surrounded by other natural and semi-natural habitats. The obtained information sheds light about the omnivory of these coccinellid and heteropteran taxa analysed, and therefore about their role as potential biological control agents. Ingestion of non-crop vegetation also indicates the role that some plants could play in attracting or maintaining these predators within both crops, important information to further improve biological control programmes.

## 2. Materials and methods

### 2.1. Sample collection and DNA extraction

Adult heteropteran and coccinellid specimens were collected in two commercial adjacent plots: one of 2 ha of peach, *Prunus persica* (L.) Batsch; and another one of 1.3 ha of alfalfa, *Medicago sativa* L., both located in Vilanova de Segrià (Lleida), Spain (UTM 10x10: 31TCGO1). They were sampled from June to September 2016, and from May to September of 2017. Peach trees were sampled close to the afternoon by beating their branches for 15 min. Alfalfa samplings were conducted using a vacuum sampler (Mc Culloch MAC320BV, Spain) in a 5 × 10 m part of the crop, situated at 2–4 m from the peach orchard also for 15 min. Each collected specimen was individualized in a DNA-free tube and placed in a portable freezer to avoid DNA degradation. Once in the lab, they were stored at −20 °C until the DNA extraction.

Before DNA extraction, all collected specimens were individually washed to remove contaminants from their cuticle, as described in Batuecas et al. (2021). Each insect and three plant samples of 1 cm diameter leaf of both *P. persica* and *M. sativa*, that were not washed were DNA extracted using the Speedtools Tissue DNA Extraction Kit (Biotools, Germany; protocol for animal tissues). Total DNA was eluted in 100 μl of AE buffer provided by the manufacturer and stored at −20 °C. A negative control without DNA (just DNA-free water) was added to each DNA extraction set. The concentration of each DNA extraction was measured using a Qubit® 2.0 fluorometer and the dsDNA HS Assay kit (Invitrogen, Carlsbad, CA, USA).

All collected specimens were used to build the sample-pools for the following HTS analysis (Table 1). For this, equimolar amounts of each individual DNA extraction (5 ng/μl) were finally pooled by species, crop, and date in 33 sample-pools (Table 1; sample-pools 1–33). In order to validate the accurately parameterized bioinformatic pipeline, three plant sample-pools built with *P. persica* and *M. sativa* DNAs, were used as positive controls (Table 1; sample-pools 34–36), as recommended by Jusino et al. (2019).

### 2.2. PCR amplification, library preparation and sequencing

The obtained sample-pools were amplified using the multi-primer approach described in Batuecas et al. (2021). For this, two pairs of universal arthropod primers were used: ZBJ-ArtF1c/ZBJ-ArtR2c (Zeale et al., 2011) and mlCOIintF/HC02198 (Leray et al., 2013; Folmer et al., 1994) that partially amplify the mitochondrial COI region, and two pairs of universal plant primers: ITS-S2F/ITS4R (Chen et al., 2010; White et al., 1990) and cA49325/trnL110R (Taberlet et al., 2007; Borsch et al., 2003), amplifying a fragment of the nuclear ITS2 region, and a fragment of the chloroplast *trnL* intron, respectively. Target DNA and DNA-free water were included as positive and negative controls, respectively. Resulting PCR products were cleaned with QIAquick PCR Purification kit (Qiagen), and 5 μl of each clean PCR product was used as template to prepare the libraries to be sequenced. HTS analysis was conducted in two batches (Table 1) and libraries were built by mixing the PCR products either of both pairs of arthropod primers, or those of both pairs of plant primers. In each batch, PCR and extraction negative controls

**Table 1**
Arthropod and plant sample-pools analysed by HTS, indicating the number of individuals or the surface of leaf included in each sample-pool, and the number of the library with the primers used.

| HTS batch number | Species/sample | Crop | Date | Sample-pool number | number of individuals | Primer pairs | Library number |
|---|---|---|---|---|---|---|---|
| 1 | *Adelphocoris lineolatus* | Alfalfa | September 2017 | 1 | 22 | ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198 | L1 |
| | | | | | | ITS-S2F/ITS4R; CA49325/trnL110R | L2 |
| | | | | 2 | 10 | ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198 | L3 |
| | | | | | | ITS-S2F/ITS4R; CA49325/trnL110R | L4 |
| | | Peach | | 3 | 6 | ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198 | L5 |
| | | | | | | ITS-S2F/ITS4R; CA49325/trnL110R | L6 |
| | *Nysius* | Alfalfa | June 2017 | 4 | 25 | ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198 | L7 |
| | | | | | | ITS-S2F/ITS4R; CA49325/trnL110R | L8 |
| | | | | 5 | 22 | ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198 | L9 |
| | | | | | | ITS-S2F/ITS4R; CA49325/trnL110R | L10 |
| | | | July 2017 | 6 | 11 | ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198 | L11 |
| | | | | | | ITS-S2F/ITS4R; CA49325/trnL110R | L12 |
| | | | September 2017 | 7 | 4 | ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198 | L13 |
| | | | | | | ITS-S2F/ITS4R; CA49325/trnL110R | L14 |
| | | Peach | June 2017 | 8 | 7 | ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198 | L15 |
| | | | | | | ITS-S2F/ITS4R; CA49325/trnL110R | L16 |
| | | | July 2017 | 9 | 3 | ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198 | L17 |
| | | | | | | ITS-S2F/ITS4R; CA49325/trnL110R | L18 |
| | | | September 2017 | 10 | 3 | ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198 | L19 |
| | | | | | | ITS-S2F/ITS4R; CA49325/trnL110R | L20 |
| | *Coccinella septempunctata* | Alfalfa | June 2016 | 11 | 6 | ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198 | L21 |
| | | | | | | ITS-S2F/ITS4R; CA49325/trnL110R | L22 |
| | | | June 2017 | 12 | 7 | ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198 | L23 |
| | | | | | | ITS-S2F/ITS4R; CA49325/trnL110R | L24 |
| | | Peach | June 2016 | 13 | 17 | ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198 | L25 |
| | | | | | | ITS-S2F/ITS4R; CA49325/trnL110R | L26 |
| | *Oenopia conglobata* | Peach | June 2016 | 14 | 5 | ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198 | L27 |
| | | | | | | ITS-S2F/ITS4R; CA49325/trnL110R | L28 |
| | | | July 2016 | 15 | 15 | ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198 | L29 |
| | | | | | | ITS-S2F/ITS4R; CA49325/trnL110R | L30 |
| | | | September 2017 | 16 | 7 | ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198 | L31 |
| | | | | | | ITS-S2F/ITS4R; CA49325/trnL110R | L32 |
| | *Stethorus punctillum* | Peach | June 2016 | 17 | 10 | ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198 | L33 |
| | | | | | | ITS-S2F/ITS4R; CA49325/trnL110R | L34 |
| | | | September 2017 | 18 | 7 | ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198 | L35 |
| | | | | | | ITS-S2F/ITS4R; CA49325/trnL110R | L36 |
| | | | | 19 | 25 | ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198 | L37 |
| | | | | | | ITS-S2F/ITS4R; CA49325/trnL110R | L38 |
| | | | | 20 | 25 | ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198 | L39 |
| | | | | | | ITS-S2F/ITS4R; CA49325/trnL110R | L40 |
| | | | | 21 | 25 | ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198 | L41 |
| | | | | | | ITS-S2F/ITS4R; CA49325/trnL110R | L42 |
| 2 | *Adelphocoris lineolatus* | Alfalfa | September 2016 | 22 | 10 | ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198 | L43 |
| | | | | | | ITS-S2F/ITS4R; CA49325/trnL110R | L44 |
| | *Lygus* | Alfalfa | July 2016 | 23 | 11 | ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198 | L45 |
| | | | | | | ITS-S2F/ITS4R; CA49325/trnL110R | L46 |

(*continued on next page*)

**Table 1** (*continued*)

| HTS batch number | Species/sample | Crop | Date | Sample-pool number | number of individuals | Primer pairs | Library number |
|---|---|---|---|---|---|---|---|
| | | | August 2016 | 24 | 8 | ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198 | L47 |
| | | | | | | ITS-S2F/ITS4R; CA49325/trnL110R | L48 |
| | | | September 2016 | 25 | 9 | ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198 | L49 |
| | | | | | | ITS-S2F/ITS4R; CA49325/trnL110R | L50 |
| | *Nabis* | Alfalfa | July 2016 | 26 | 23 | ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198 | L51 |
| | | | | | | ITS-S2F/ITS4R; CA49325/trnL110R | L52 |
| | | | August 2016 | 27 | 14 | ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198 | L53 |
| | | | | | | ITS-S2F/ITS4R; CA49325/trnL110R | L54 |
| | | | June 2017 | 28 | 16 | ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198 | L55 |
| | | | | | | ITS-S2F/ITS4R; CA49325/trnL110R | L56 |
| | | | July 2017 | 29 | 25 | ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198 | L57 |
| | | | | | | ITS-S2F/ITS4R; CA49325/trnL110R | L58 |
| | *Coccinella septempunctata* | Alfalfa | May 2017 | 30 | 7 | ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198 | L59 |
| | | | | | | ITS-S2F/ITS4R; CA49325/trnL110R | L60 |
| | *Hippodamia variegata* | Alfalfa | June 2016 | 31 | 7 | ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198 | L61 |
| | | | | | | ITS-S2F/ITS4R; CA49325/trnL110R | L62 |
| | | | July 2016 | 32 | 16 | ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198 | L63 |
| | | | | | | ITS-S2F/ITS4R; CA49325/trnL110R | L64 |
| | | | August 2016 | 33 | 25 | ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198 | L65 |
| | | | | | | ITS-S2F/ITS4R; CA49325/trnL110R | L66 |
| 1 | *Prunus persica* | Peach | – | 34 | 1 $cm^2$ | ITS-S2F/ITS4R; CA49325/trnL110R | L67 |
| | *Medicago sativa* | Alfalfa | – | 35 | 1 $cm^2$ | ITS-S2F/ITS4R; CA49325/trnL110R | L68 |
| 2 | *Medicago sativa* | Alfalfa | – | 36 | 1 $cm^2$ | ITS-S2F/ITS4R; CA49325/trnL110R | L69 |
| 1 | PCR blank | – | – | 37 | – | ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198 | L70 |
| | | | | | | ITS-S2F/ITS4R; CA49325/trnL110R | L71 |
| 2 | PCR blank | – | – | 38 | – | ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198 | L72 |
| | | | | | | ITS-S2F/ITS4R; CA49325/trnL110R | L73 |
| | Extraction blank | – | – | 39 | – | ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198 | L74 |
| | | | | | | ITS-S2F/ITS4R; CA49325/trnL110R | L75 |

were included (Table 1, sample- pools 37, 38 and 39). Both batches were processed on a MiSeq sequencing platform (Illumina, San Diego, CA, USA) at the *Servei de Genòmica i Bioinformàtica* of the Autonomous University of Barcelona, Spain. Illumina adapters were attached using Nextera XT Index kit. Amplicons were purified with magnetic beads and 5 μl of each library were grouped and sequenced with a paired-end approach (2 X 225 bp).

### 2.3. Bioinformatics

We performed the same steps of the bioinformatic analysis described in Batuecas et al. (2021). Raw Illumina reads were merged, and assembled reads were filtered for quality with a minimum of 75 % of bases ≥ Q30. Resulting reads were split by length and clustered into OTUs with a similarity threshold of 97 %. Chimeras were removed and the remaining OTUs were queried against custom-made databases with restrictive parameter for BLAST (BLASTN, E-value 1e-10, minimum coverage of the query sequence: 97 %, numbers of alignments: 9). Taxonomy was assigned at ≥97 % identity by Last Common Ancestor algorithm (LCA) with BASTA (Kahlke & Ralph, 2019). To remove possible contaminants, we only considered those OTUs that strictly had more than five reads and were detected in at least two sample-pools (Boyer et al., 2013). In those cases where the OTUs were obtained in only one sample-pool, they were considered for the analysis if they had an abundance of more than five reads with both primer pairs or if they exceeded the 0.03 % threshold of the total reads from OTUs filtered for plant or arthropod in each case.

To reduce biases not eliminated before, or spurious results as secondary predation, and with the aim of showing the most important taxa ingested, diet data were represented using two dietary metrics, as recommended by Deagle et al. (2018). The first metric was the percentage of Relative Read Abundance (RRA%), which was calculated from the total number of reads of each consumed resource group (arthropod or plant) amplified with each primer pair and for each library, divided by the number of total reads of all resources obtained with each primer pair for each library. After that, a new filter was applied, which eliminated those resources with a value of RRA < 2.5 %, which was even higher than the 1 % threshold recommended by Deagle et al. (2018). With the taxa obtained, a second metric was calculated, which was the percentage of Frequency of Occurrence (FOO%), being the percentage of the

number of pools of each analysed specimens that contain a particular resource.

## 3. Results

### 3.1. Predator identification

A total of 433 heteropteran (*Adelphocoris lineolatus* Goeze, *Nysius* sp., *Lygus* sp. and *Nabis* sp.) and coccinellid (*Coccinella septempunctata* L., *Hippodamia variegata* Goeze, *Oenopia conglobata* L. and *Stethorus punctillum* Weise) specimens were collected in both peach and alfalfa crops. They were morphologically identified to species level, except *Nysius*, *Lygus* and *Nabis*, that were identified to genus level. Taxa varied according with the crop and the sampling date. *Adelphocoris lineolatus, Nysius* and *C. septempunctata* were found in both crops; *Lygus* and *Nabis* were found only in alfalfa; and *O. conglobata* and *S. punctillum* were only found in peach.

### 3.2. HTS analysis of field-collected predators

The HTS analysis of the 75 libraries (Table 1) generated 17,022,304 raw paired-end reads. The number of reads and OTUs obtained in each step of the bioinformatic process is reported in Table S1. From the raw paired-end reads obtained in step 0, only 8930 (0.039 %) came from the DNA extraction blank (Table 1, sample-pool 39) and both PCR blanks (sample-pools 37 (batch 1) and 38 (batch 2) (Table 1). After step 1, 97.55 % of the initial reads were successfully merged, discarding 207,704 raw reads. After that, 207,704 (step 2) and 161,548 (step 3) reads were discarded, respectively. After clustering the reads (step 4), 62,619 chimera reads were discarded (step 5). After the taxonomy assignment (step 6), 2270 arthropod and 828 plant OTUs were filtered (step 7). After the OTUs filtering (step 7) to eliminate contaminants (the reads obtained from both PCR blanks and the DNA extraction blank), the taxa with RRA lower than 2.5 % were also eliminated (Table S1 step 8; Table S2). From the 39 sample-pools analysed (Table 1), we obtained 795 arthropod and 210 plant OTUs, which were finally assigned to 27 arthropod taxa (17 to species level) and 14 plant taxa (four to species level) (Table 2, A and B). The predator itself was detected in all analysed sample-pools, either to genus or to species level (Table S3).

The three analyzed taxa that were collected in both alfalfa and peach: *A. lineolatus* (sample-pools 1, 2, 3 and 22, Table 1), *Nysius* (sample-pools 4 to 10, Table 1), and *C. septempunctata* (sample-pools 11, 12, 13 and 30, Table 1), showed arthropod and plant amplification in all sample-pools (Table S3). Regarding those predators collected in alfalfa, from *A. lineolatus* we detected one pest species (*Nysius graminicola* Kolenati & F.A. (Lygaeidae)); five predator taxa belonging to two families (Coccinellidae and Miridae), one genus (*Adelphocoris)* and two species (*O. conglobata* and *Rhagonycha fulva* Scopoli (Cantharidae)); one non-pest species (*Diaphorina lycii* Loginova (Liviidae)); and six plant taxa corresponding to *Medicago sativa* L. (the crop where they were collected), some unidentified plants of the Clade Streptophyta, three families (Asteraceae, Fabaceae and Poaceae), and one species (*Beta vulgaris* L. (Amaranthaceae)) (Table 2). From the *Lygus* genus, which was the other mirid bug collected in alfalfa (sample-pools 23 to 25, Table 1), arthropod and plant amplification was obtained in all analysed sample-pools (Table S3). *Lygus* did prey on the same pest species than *A. lineolatus* (*N. graminicola*), as well as on Streptophyta and three families (Asteraceae, Fabaceae and Solanaceae) (Table S3; Fig. 1 and Fig. 2). Those *Nysius* collected in alfalfa, were identified as two different species: *N. graminicola* and *Nysius cymoides* Spinola. They consumed three arthropod taxa, corresponding to two families (Coccinellidae and Miridae) and one species (*R. fulva*); and three plant families (Asteraceae, Fabaceae and Solanaceae). The analysis of the diet of the *Nabis* specimens, which were only collected in alfalfa (sample-pools 26 to 29, Table 1), showed arthropod and plant amplification in all tested sample-pools (Table S3). We detected five pest taxa, corresponding to two genus (*Aphis* and *Hypera* (Curculionidae)) and three species (*Aphis craccivora* Koch & C.L., *Therioaphis trifolii* Monell (Aphididae), and *N. graminicola*); six predator taxa (two families (Cecidomyiidae and Miridae), one genus (*Sphaerophoria* (Syrphidae)), and three species *(Aeolothrips intermedius* Bagnall (Aeolothripidae), *Deraeocoris serenus* Douglas & Scott (Miridae) and *Orius niger Wolff* (Anthocoridae)). We also detected one parasitoid, the tachinid *Leucostoma* sp., known to parasitize Heteroptera (like *Nabis*), as well as three plant taxa (*M. sativa* and two families (Asteraceae and Fabaceae) (Table S3; Fig. 1 and Fig. 2; Table 2). From those *C. septempunctata* collected in alfalfa, the following arthropods were detected: two pest taxa corresponding to one genus (*Aphis*) and one species (*N. graminicola*); the coccinellid parasitoid *Dinocampus coccinellae* Schrank (Braconidae) (probably because they were parasitising the analized predators); two predator taxa belonging to one family (Coccinellidae) and one species (*R. fulva)*; and five plant taxa

**Table 2**
Summary table of all detected arthropod (A; n = 27) and plant (B; n = 14) taxa after the bioinformatic analysis of HTS data from 75 libraries of 39 different sample-pools described in the Table 1.

| A | |
|---|---|
| Taxonomic level | Taxa detected |
| Family | Coccinellidae<br>Cecidomyiidae<br>Miridae |
| Genus | *Aphis*<br>*Adelphocoris*<br>*Hypera*<br>*Leucostoma*<br>*Lygus*<br>*Nabis*<br>*Sphaerophoria* |
| Species | *Adelphocoris lineolatus* Goeze<br>*Aeolothrips intermedius* Bagnall<br>*Aphis craccivora* Koch & C.L.<br>*Cantharis livida* L.<br>*Coccinella septempunctata* Linnaeus<br>*Deraeocoris serenus* Douglas & Scott<br>*Diaphorina lycii* Loginova<br>*Dinocampus coccinellae* Schrank<br>*Hippodamia variegata* Goeze<br>*Lipolexis gracilis Forster*<br>*Nysius cymoides* M.Spinola<br>*Nysius graminicola* Kolenati<br>*Oenopia conglobata L.*<br>*Orius niger* Wolff<br>*Rhagonycha fulva* Scopoli<br>*Stethorus punctillum* Weise<br>*Therioaphis trifolii* Monell |

| B | |
|---|---|
| Taxonomic level | Taxa detected |
| Phylum | Streptophyta |
| Order | Caryophyllales |
| Family | Asteraceae<br>Brassicaceae<br>Fabaceae<br>Poaceae<br>Rosaceae<br>Solanaceae |
| Genus | *Malva*<br>*Pinus* |
| Species | *Beta vulgaris L.*<br>*Medicago sativa* L.<br>*Poa annua* L.<br>*Prunus persica* (L.) Batsch |

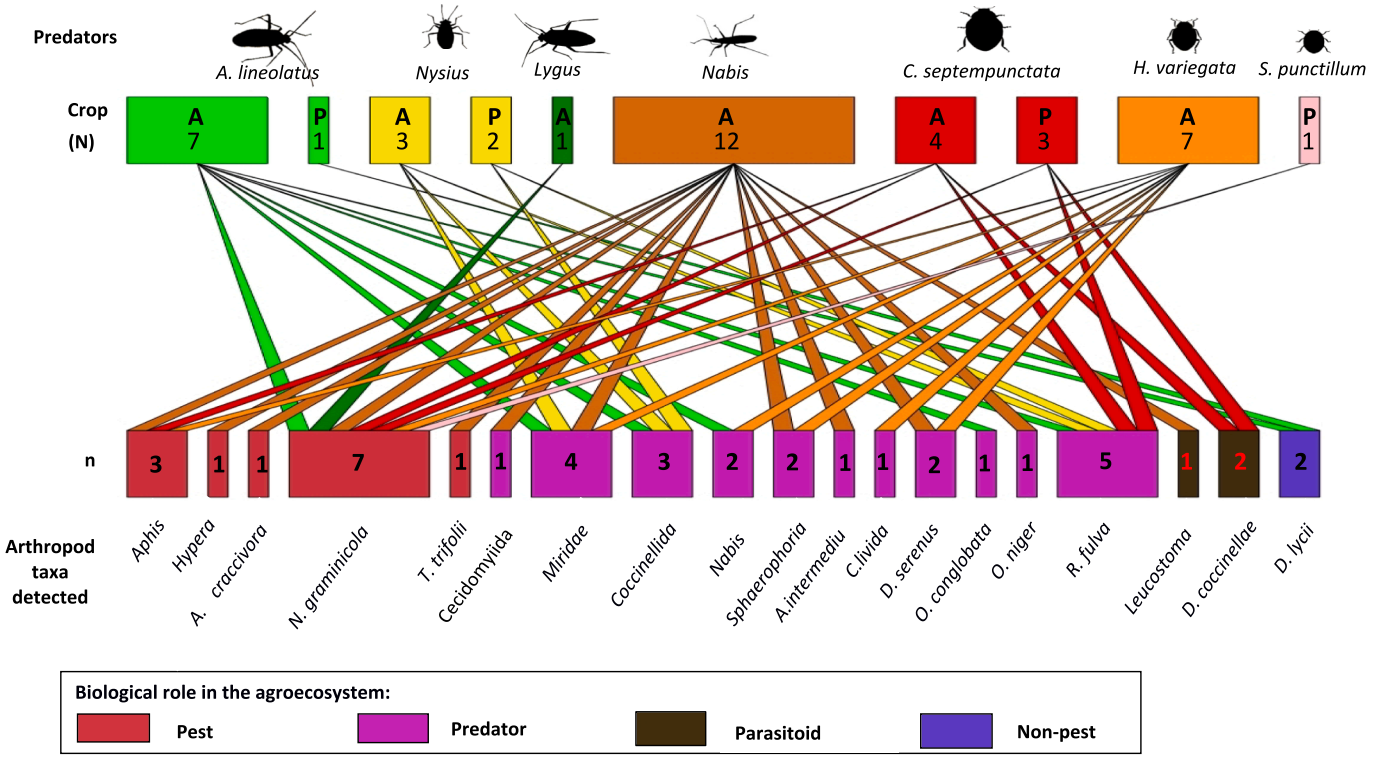


**Fig. 1.** Representation of the number of detected arthropod interactions for each analysed predator (*A. lineolatus, Nysius, Lygus, Nabis, C. septempunctata, H. variegata* and *S. punctillum*) collected in alfalfa (**A**) and peach (**P**). N = number of arthropod taxa detected from each predator analysed. n = Number of predators that consumed the arthropod taxa detected.

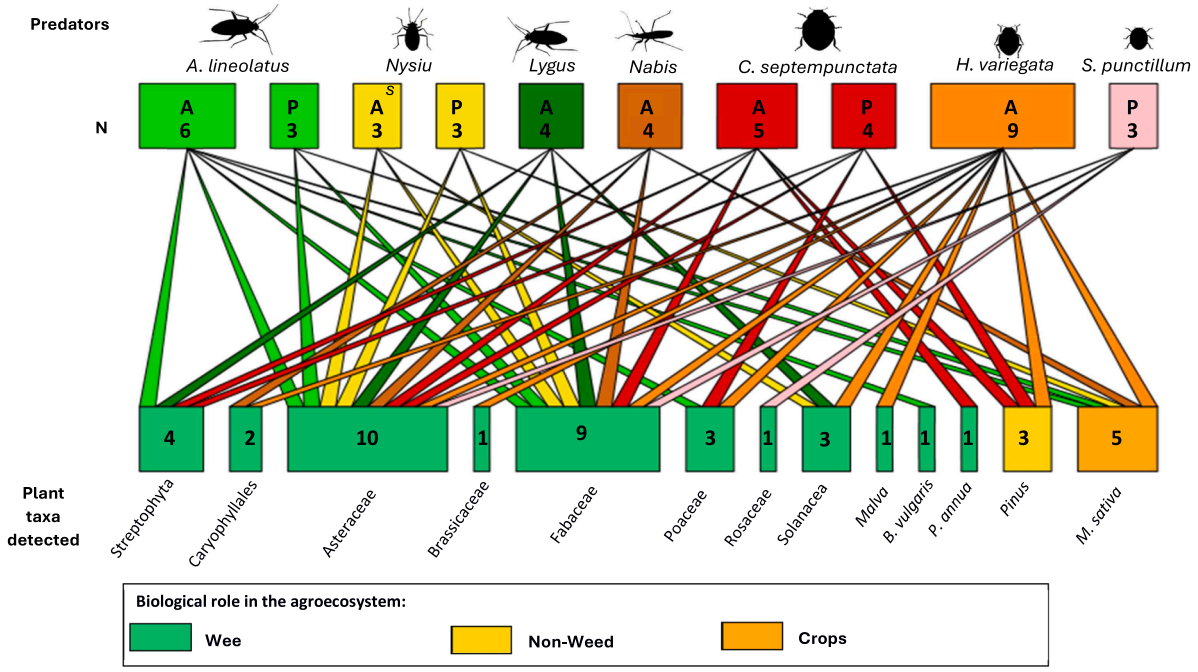


**Fig. 2.** Representation of the number of detected plant interactions for each analysed predator (*A. lineolatus, Nysius, Lygus, Nabis, C. septempunctata, H. variegata* and *S. punctillum*) collected in alfalfa (**A**) and peach (**P**). N = number of plant taxa detected from each predator analysed. n = Number of predators that consumed the plant taxa detected.

(Streptophyta, two families (Asteraceae and Fabaceae), one genus (*Pinus*) and one species (*Poa annua* L. (Poaceae)). Regarding *Hippodamia variegata*, only found in alfalfa too (sample-pools 31 to 33, Table 1), arthropod and plant amplification was obtained in all the analysed sample-pools. We detected two pest taxa (the genus *Aphis* and the species *N. graminicola*); six predator taxa (two families (Coccinellidae and Miridae), one genus (*Nabis*), and three species (*C. livida*, *D. serenus* and *R. fulva*); and ten plant taxa (Streptophyta, one order (Caryophyllales),

five families (Asteraceae, Brassicaceae, Fabaceae, Poaceae and Solanaceae), two genera (*Malva* and *Pinus*) and the species *M. sativa* (Table S3; Fig. 1 and Fig. 2).

Regarding those predators collected in peach, from *A. lineolatus* we detected one arthropod non-pest taxon (*D. lycii*) and three plant taxa (one corresponding to alfalfa (*M. sativa*), and two plant families (Asteraceae and Fabaceae) (Table S3, Fig. 1, Fig. 2). From *Nysius* we identified only one *Nysius* species (*N. graminicola*), and we detected two other arthropod taxa, corresponding to one family (Coccinellidae) and one species (*R. fulva);* and three plant taxa, one species (*M. sativa*) and two families (Asteraceae and Fabaceae) (Table S3; Fig. 1 and Fig. 2). From *C. septempunctata* we detected one pest species (*N. graminicola*); one parasitoid (*D. coccinellae);* two predator taxa corresponding to one family (Coccinellidae) and one species *(R. fulva*); and four plant taxa (Streptophyta), two families (Asteraceae and Poaceae), and one genus (*Pinus*) (Table S3; Fig. 1 and Fig. 2). *Oenopia conglobata,* which was only found in peach (sample-pools 14 to 16, Table 1), just amplified the predator itself. No other arthropods or plants were detected (Table S3; Fig. 1 and Fig. 2). *Stethorus punctillum,* which was also only found in peach (sample-pools 17 to 21, Table 1), showed arthropod and plant amplification in all sample-pools. We detected two arthropod taxa corresponding to one family (Coccinellidae) and one pest species (N. *graminicola*), as well as three plant families (Asteraceae, Fabaceae and Rosaceae) (Table S3; Fig. 1 and Fig. 2).

Regarding the plant positive control of peach (sample-pool 34; Table 1) and alfalfa (sample-pools 35 and 36; Table 1), we amplified *P. persica* and *M. sativa*, respectively, as well as their corresponding families (Rosaceae and Fabaceae) (Table S3). Rosaceae and Streptophyta were also detected in sample-pools 34 and 35 (Table S3). Nevertheless, they represented only 0.0056 % of the total plant reads obtained after applying all filters. For this reason, they were not considered in further analysis.

## 4. Discussion

This study was performed to assess the most common trophic interactions of some heteropterans and coccinellids in a peach and an alfalfa adjacent crop by an HTS multi-primer metabarcoding approach. Results showed this method as a reliable tool to understand the trophic interactions of these two groups of insects present in this agroecosystem, and to elucidate their role as omnivorous predators. This methodology was also able to detect potential pests of both crops, to detect intraguild predation (IGP) among predators (including those analysed), to show the importance of some plant species as predator resources, and to demonstrate insect movement between elements of the landscape, particularly from the field margins to the crops.

### 4.1. Methodological issues

HTS techniques allow amplifying the ingested taxa (Forin-Wiart et al., 2018). In the present study, we have performed the HTS analysis by using sample-pools in order to increase the robustness of the results. Those predator species collected in the same crop and date were grouped in sample-pools, which were used as biological replicates (Table 1). It has been described that pooling samples improves the detection of the most consumed species, avoiding the detection of spurious trophic interactions with less importance (Mata et al., 2019). We prioritized the use of biological replicates instead of technical replicates of the PCR reactions because biological replicates in HTS led to obtain a more significant variation in the prey species composition than those obtained by different PCR replicates per sample (Mata et al., 2019). The analysis of a single PCR replicate per sample, like in the present study, allows the identification of the most abundant ingested resources in a sample-pool (Leray & Knowlton, 2017). Therefore, the use of biological replicates indicates that if a taxon is detected in all sample-pools, it must be a common resource, as previously done in Batuecas et al. (2021; 2022).

In the present study, we built our own customed database with sequences downloaded from the NCBI public database, including those taxa potentially present in the agroecosystem where the study was performed. It is well known that some errors could be present in those sequences (Shen et al., 2013), and that the absence of sequences for some species potentially present in the studied ecosystems is frequent (Corse et al., 2019). The completeness and reliability of the databases are the most critical limitations of metabarcoding studies (Galan et al., 2018). The absence of sequences in those databases generate false negative results, not detecting the occurrence of those species not included in the databases (Sow et al., 2019). For these reasons, an exhaustive and customized database is key to the reliability from the results.

The restrictive sequence analysis conducted in the present study shows that 85 % and 73 % of the OTUs obtained of arthropods and plants, respectively, were not identified after the assignment using our own customed database (Table S1, step 6). This high percentage of unassigned OTUs is probably due to the scarcity of representative sequences of arthropods and plants from the Ebro basin in the NBCI database. After this assignment and the use of both filters (Steps 7 and 8), the obtained trophic interactions came finally from the 25 % of the obtained OTUs. Nevertheless, this still gives a good overview of the main trophic interactions. Caution should be taken to discriminate between primary and secondary predation (hyperpredation) in metabarcoding studies, that is to say, when one predator feeds on another one that has recently eaten another prey, or when the predator is parasitized. This has been reported as one of the most important limitations of the HTS technique (Galan et al., 2018; Da Silva et al., 2019). To decrease its impact, we have calculated the RRA% of the detected taxa, as done in Batuecas et al. (2022). In the present study we detected secondary predation of *Lipolexis gracilis* Förster (Hymenoptera: Braconidae) in two *Nabis* sample-pools (25 and 27, Table 1) with an RRA of 2.29 % and 1.09 %, respectively (Table S2(2)). *Lipolexis gracilis* is a primary parasitoid of Aphididae, which included *A. craccivora* in alfalfa (Pons et al., 2011), being evident in this case that *Nabis* indirectly ingested it. To avoid this secondary predation detection, we decided to apply a threshold of 2.5 % in the bioinformatic analysis. The detection of this parasitoid in *Nabis* could be because this parasitoid is one of the most prevalent in alfalfa in the area of study (Pons et al., 2011). The high FOO% of *Aphis* and *A. craccivora* (75 % *Aphis*, 100 % *A. craccivora*; Fig. S1A) in the *Nabis* samples indicated that this predator prefers aphids, and that they were very common in the agroecosystem, which could also favor the presence of the parasitoid. *Lipolexis* spp. have also been reported in the same peach plot parasitizing *Myzus persicae* (Sulzer) (Aparicio et al., 2019; Kocić et al., 2020).

Our results also showed that the methodology used is also helpful to distinguish between cryptic species, like *N. graminicola* and *N. cymoides* in the *Nysius* sample-pools (Table S3). However, in other cases, we did not identify different species of a genus, as in the *Lygus* or *Nabis* analyzed sample-pools. The explanation could be the lack of sequences in the public databases, as previously stated. The genus *Lygus* is represented in the study area mainly by *Lygus rugulipennis* Poppius (López-Marin et al., 2017), but also by *Lygus pratensis* L., *Lygus punctatus* Remane and *Lygus wagneri* Remane. The genus *Nabis* is represented by *Nabis pseudoferus* Remane, *Nabis provencalis* Remane and *Nabis punctatus* A. Costa in the same area of study (Nuñez, 2002; Pons & Eizaguirre, 2009). When we built the customized database, the available sequences in the NCBI database for *Lygus* were *L. rugulipennis*, *L. pratensis*, *L. punctatus*, *L. lineolaris* and *L. gemellatus*. However, the analysed specimens could also belong to *Lygus italicus* Wagner o *Lygus maritimus* Wagner, as they had also been cited in Nord-Eastern Spain (Goula et al., 2020). Regarding *Nabis*, the available sequences when the analyses were conducted belonged to *N. pseudoferus* and *N. punctatus*. Then, it is possible that the anaylsed specimens belonged to *N. provencalis*, the most abundant in the area of study in alfalfa (Nuñez, 2002), but because its sequence is not available in the NCBI database, we have not detected it.

In the HTS analysis, most of the detected taxa (70 %) were identified to genus/species level, which is a suitable taxonomic resolution according to a previous HTS study of omnivory conducted with mammals, that reached 60 % (De Barba et al., 2014). The obtained taxonomic resolution agrees with the results obtained in Batuecas et al. (2021; 2022), detecting mainly species when using COI or ITS2 regions, and mainly families when using the *trnL* plant region, as happens in the present study and in Elbrecht et al. (2016) and De Barba et al. (2014).

### 4.2. Trophic interactions

The HTS multi-primer approach used showed the omnivory of all heteropteran and coccinellid analysed, except for *O. conglobata,* from which no arthropod or plant resources were detected. Even if this species is a well-known predator in herbaceous plants (Hodek & Honek, 1966, Mehrnejad & Jalali, 2004, Lumbierres et al., 2018), we did not detect a feeding episode of this species. A part of this species, the taxa amplified by both arthropod and plant primer pairs showed a certain food web structure with species feeding on different trophic levels (phytophagous, other predators, parasitoids and plants) (Fig. 1 and Fig. 2). This food web is represented by 18 trophic interactions, five on pest taxa, ten on polyphagous predators and two on parasitoids (*Leucostoma* and *D. coccinellae*) (Table S2), showing that in fact, the most detected trophic interactions were related to IGP, as it shows these 13 of the 18 detected arthropod taxa. This IGP included *D. serenus*, *Nabis* and *O. conglobata,* as well as *R. fulva*, *O. niger, C. livida* and *A. intermedius*, four predatory taxa also detected in previous studies conducted in the same area of study (Batuecas et al., 2021; 2022). Other previous HTS studies conducted in other agroecosystems have also detected parasitism (Lefort et al., 2017; Sow et al., 2019). Regarding *Leucostoma*, several species are present in southern Europe, including the Ebro basin, like some heteropteran parasitoids, as well as *Nabis* parasitoids in alfalfa (Lattin, 1989; Tschorsnig, 1992). Our detection of parasitism of *C. septempunctata* by *D. coccinellae* is congruent with field observations conducted by Pons et al. (2015).

From the four heteropteran taxa analysed, three of them (*A. lineolatus, Nysius* and *Lygus*) have been traditionally considered as phytophagous, being either pests of alfalfa or peach (Del Rivero & García-Marí, 1983; Schaber and Entz, 1994; Blando & Mineo, 2005). Our results demonstrated for the first time their omnivory, preying on pests (*N. graminicola*), other predators (*Nabis, O. conglobata* and *R. fulva*), and non-pest prey (*D. lycii*) (Fig. 1). Some of these predation episodes had been suggested by Nuñez (2002), based on field observations of *A. lineolatus* and *Lygus,* but no information is available in the literature regarding predatory episodes of *Nysius.* This genus is mainly composed of secondary pests of different crops, like olive, grapevine, or tomato, being also present in a wide range of other plant species (Scacinni and Fuland, 2019). In the present study, *A. lineolatus* fed on the non-pest species *D. lycii*, being the most common trophic interaction of this predator (Fig. S1A). *Diaphorina lycii* is oligophagous on *Lycium* plants (Solanaceae) (Burckhardt, 1984), and *Lycium europaeum* L. is commonly planted within hedges and as hedges rows to separate agricultural plots in the study area (Bolòs and Vigo, 1996). Therefore, it could be assumed that *A. lineolatus* have moved from those plants located in the margins to the peach crop and to the alfalfa crop. Probably they find more plant resources there, both in the crops themselves or in the plants that are present in the ground covers. In previous HTS studies, movements of other heteropterans have also been detected among other elements of the landscape in the same area of study, like *Anthocoris nemoralis* Fabricius moving from the field margins or from other more distant plants to the peach crop (Batuecas et al., 2021), or *Orius* spp. moving from peach to alfalfa (Batuecas et al., 2022). *Nabis* showed the highest number of trophic interactions (Fig. 1), confirming its predatory nature. This genus is a known biological control agent in alfalfa (Pons et al., 2009). In the present study it was detected to prey on some alfalfa pests, like some Aphididae (*Aphis, A. craccivora* and *T. trifolii*) and *Hypera*, trophic interactions already cited by Nuñez (2002) and Pons et al. (2009). On the other hand, *Nabis* was also detected to prey on *N. graminicola*, as well as on other predatory taxa (Fig. 1).

Regarding the four analysed coccinellid taxa, in three of them some crop pests, like aphids or *N. graminicola,* were detected (Fig. 1). *Coccinella septempunctata* and *H. variegata* collected in alfalfa showed their well-known aphidophagous nature, already cited by Pons et al. (2009) in the same area of study. Nevertheless, the most important trophic interaction was on *N. graminicola,* which was detected in seven of the eight analysed predator species (Fig. 1). This species has been cited to be an important pest of several summer crops in Italy, including peaches (Blando & Mineo, 2005). *Nysius graminicola* has been also described in citrus in Catalonia, but it has not been considered as a crop pest (Ribes et al., 2004). On the other hand, *N. cymoides* was also detected in two sample-pools (Table S3). This species causes damages in some crops in the Middle East and Europe, including alfalfa (Scaccini & Furlan, 2019). Its presence in the area of study had not been documented until now.

Plant DNA was detected in all analysed heteropterans and coccinellids (except *O. conglobata*) both sampled years, showing their omnivorous trend. The most consumed plant taxa were Asteraceae, Poaceae, Solanaceae, Fabaceae and *M. sativa* (Fig. S1C and D; Fig. 1B). These predators were collected in summer, when green plants were scarce in the field margin and ground covers. In the area of study, all plants are dry at this time of the year because of the extremely high temperature, and the only green plant at that time is alfalfa, together with the ground cover of the irrigated peaches (Clemente-Orta et al., 2021). The same plant taxa were obtained when *R. fulva* and *Orius* spp. collected in the same area of study and at the same time of the year were analysed using the same kind of HTS analysis (Batuecas et al., 2021; 2022), emphasizing the importance of these plant taxa for several predator species present in the studied agroecosystem. As shown in Figure S1(C and D), some plant taxa showed a high FOO%, meaning that were important in the diet of the analysed predators, like Caryophyllales, Brassicae, *Malva* and *Pinus* for *Nabis; B. vulgaris* for *A. lineolatus*; and Caryophyllales, Brassicae, *Malva* and *Pinus* for coccinellids. These results seem to indicate that different groups of predators may use these plants by directly feeding on the plant or the shed pollen. The dietary metrics RRA% and FOO% showed more reliable evidence of consumption by giving an estimation from the reads obtained in each sample and the frequency that the taxa are detected in the analysed samples, demonstrating that consumption on a particular taxon is not spurious or not indirectly ingested (secondary predation). Therefore, these plants should be further considered when establishing flower margins for predator conservation or their attraction to the crop. *Pinus* was detected within *C. septempunctata* collected in alfalfa and peach crops, and within *H. variegata* from alfalfa. The same was observed in a previous study with *Orius* collected in the same alfalfa and peach field plots (Batuecas et al., 2022). This detection can be only explained by the presence of wind spread *Pinus* pollen deposited on both crops, because pine trees were not very close to the studied plots. *Pinus* pollen could have a significant effect on these biological control agents as a secondary resource, particularly in this challenging period of the year when plant resources are so limited. The use of wind spread pollen have been previously demonstrated by predatory mites that used maize pollen (González-Fernández et al., 2009).

This study showed the omnivorous role of the four heteropteran (*A. lineolatus, Nysius, Lygus* and *Nabis*) and three coccinellid (*C. septempunctata*, *H. variegata* and *S. punctillum*) taxa present in an agroecosystem composed by a peach and an alfalfa plot in the Ebro Basin. The most common trophic interactions also elucidate the movement of these insects between neighboring habitats. The detection of predator movement between crops, as well as from other plants located in the margins of those crops demonstrate the importance of the landscape biodiversity. This information could be useful to improve pest management in agroecosystems enhancing biological control programs in these crops. The detection of *N. cymoides,* a potential pest in peach

crops should be further investigated, showing the potential of this tool also for biomonitoring the presence of new threats.

## CRediT authorship contribution statement

**Ivan Batuecas:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Oscar Alomar:** Writing – original draft, Supervision, Investigation, Funding acquisition, Conceptualization. **Cristina Castañe:** Writing – original draft, Supervision, Investigation, Conceptualization. **Nuria Agustí:** Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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### *Author Statement*

IB and NA conceived and designed the HTS analysis and wrote the manuscript. IB and NA conducted de HTS analysis. IB, CC, OA and NA conducted the field samplings. IB analysed the data. All authors revised and approved the manuscript.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biocontrol.2024.105545.

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