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Research Article

Invasive plant-induced shifts in water chemistry and microeukaryotes enhance mosquito development

Oriol Cano-Rocabayera[1,2,3], Ydsell Y. Bonilla[1], Humbert Salvadó[4], Marc Garcia-Bargalló[4], Islam S. Sobhy[5,6], Colin Berry[5], Montserrat Vilà[7,8], Francesc Sabater[1], Carles Aranda[9,10], Alberto Maceda-Veiga[1,11]

1 FORESTREAM Research Group, Departament de Biologia Evolutiva, Ecologia i Ciències Ambientals, Facultat de Biologia, Universitat de Barcelona (UB), Barcelona, Spain
2 Aquatische Ökotoxikologie Department, Goethe Universität Frankfurt, Max-von-Laue Str. 13, Frankfurt am Main, Germany
3 Center for Environmental and Human Toxicology, University of Florida, Gaineville, USA
4 Virus, Bacteria, and Protozoa Research Group, Departament de Biologia Evolutiva, Ecologia i Ciències Ambientals, Facultat de Biologia, Universitat de Barcelona, Barcelona, Spain
5 School of Biosciences, Cardiff University, Cardiff, UK
6 Current address: School of Natural Sciences, University of Chester, Chester, CH1 4BJ, UK
7 Departamento de Biología de la Conservación y Cambio Global, Estación Biológica de Doñana (EBD-CSIC), Sevilla, Spain
8 Department of Plant Biology and Ecology, Universidad de Sevilla, Sevilla, Spain
9 Servei de Control de Mosquits del Consell Comarcal del Baix Llobregat, El Prat de Llobregat, Spain
10 IRTA, Centre de Recerca en Sanitat Animal (CReSA, IRTA-UAB), Campus de la Universitat Autònoma de Barcelona, Bellaterra, Spain
11 Institut de Recerca de la Biodiversitat (IRBio), Facultat de Biologia, Universitat de Barcelona, Barcelona, Spain
Corresponding author: Alberto Maceda-Veiga (albertomaceda@gmail.com)

Abstract

Invasive non-native plants can cause ecological, economic, and health impacts worldwide, yet how these impacts cascade from a primary producer to multiple consumers remains poorly understood. Using aquatic microcosms, we examined how replacing the native reed *Phragmites australis* with the morphologically similar invader *Arundo donax* (0–100% leaf litter biomass gradient) alters water physicochemistry, microeukaryote assemblages, and the development of *Culex pipiens* mosquito larvae in northeastern Spain. Microeukaryotes are key players in microbial food webs, and mosquito larvae are microbial consumers. Increased *A. donax* leaf litter % disrupted microeukaryote taxonomic composition and functional guild structure, mainly due to changes in the density of flagellates, planktonic ciliates, and micrometazoa. Mosquito larval development was mainly driven by changes in water physicochemistry, flagellates, and amoebae. Microcosms with *A. donax* produced more mosquito pupae of greater weight and shorter development times. Effects emerged even when only 25% of *P. australis* biomass was replaced by *A. donax*, suggesting potentially strong nutrient limitations from *P. australis* leaf litter and posing challenges for defining a management “safe threshold” for *A. donax* when eradication is unfeasible. This study highlights *A. donax* leaf litter as a potential promoter of mosquito development and underscores the role of water chemistry and microeukaryotes in mediating its effects.

Key words: *Arundo donax*, *Culex pipiens*, metals, *Phragmites australis*, protozoa assemblages, riparian leaf litter, trophic cascade, vector ecology

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Introduction

Invasive non-native plants cause multitrophic impacts in invaded ecosystems through direct interactions with native species and alterations in habitat structure or environmental conditions (Gallardo et al. 2016; Weidlich et al. 2020; Vilà et al. 2024). The consequences of these impacts can be extensive, ranging from the loss of native species and alterations in nutrient cycling to effects on human well-being (Bonanomi et al. 2006; Vilà and Hulme 2017). However, our understanding of how these impacts cascade through a food web remains limited because most studies have focused on a single or just two trophic levels (Ehrenfeld 2003; Hulme et al. 2013). This knowledge gap impedes our ability to predict the effects of non-native plant invasions on the structure and function of ecosystems.

Leaf-litter decomposition is a key ecosystem process through which invasive non-native plants exert multitrophic impacts (Ehrenfeld 2003). While the role of bacteria and fungi is well documented, that of microeukaryotes, including protozoa and small metazoans (e.g., rotifers, nematodes, and gastrotrichs), has been largely overlooked (Foissner 1999; Reiss 2021), despite their significant contribution to the organic matter living on leaf litter (Brüchner-Hüttemann et al. 2019). Leaf litter of invasive non-native plants may disrupt microeukaryote assemblages through the release of chemicals such as ammonia and toxic metals, to which microeukaryotes can be particularly vulnerable (Foissner 1999; Maceda-Veiga et al. 2022). However, the seemingly functional redundancy of microbial food webs may replace the function of the lost taxa without affecting upper trophic levels (Custer and van Diepen 2020; Torres et al. 2021). Therefore, the multitrophic impacts of invasive non-native plants may be better understood if studies examine simultaneously the performance of a consumer and the taxonomic and functional structure of microeukaryote assemblages.

The structure of aquatic food webs in which leaf litter is a primary energy source is highly variable, given the diversity of natural and human-made habitats involved, including tree holes, irrigation ponds, wetlands, and lowland rivers, among many others (Stoler and Relyea 2013; González et al. 2014; Ferreira et al. 2016). However, all these habitats share the common feature, in the warm season, that mosquito larvae often play a significant role as predators of leaf-litter microbes, including microeukaryotes (González et al. 2014; Yee and Juliano 2006; Skiff and Yee 2015). In fact, in small water bodies or in waters severely polluted with reduced oxygen levels, mosquito larvae can become the primary apex consumer due to their ability to breathe air (Walker et al. 2010; Becker et al. 2010). Beyond the role of mosquitoes as pollinators and prey for insectivores, the development of mosquito larvae can impact public health because adult mosquitoes can be vectors for many pathogens (Becker et al. 2010; Brugueras et al. 2020). This explains why research interest in the influence of landscape factors such as vegetation structure on mosquito populations is growing (Ferraguti et al. 2016; Ferraguti et al. 2023; Chikodza et al. 2025), but little attention has been given to the contrasting effects of leaf litter from native and invasive non-native plants on mosquito development (Gardner 2016; Cuthbert et al. 2019). While vegetation alone is unlikely to be sufficient to control mosquito populations, identifying whether there are plants that can either accelerate or reduce mosquito larval development can help authorities pinpoint particularly high-risk habitats when targeting mosquito breeding sites.

The invasive giant reed *Arundo donax* Linnaeus, 1753, forms dense stands in Mediterranean streams, ponds, ditches, and other habitats suitable for mosquitoes (Becker et al. 2010), replacing key native plant species such as *Phragmites australis* Cav.Trin. ex Steud., 1840 (Resolution n°4 1996; Čížková et al. 2023) and altering the abundance and diversity of consumers (Dudley 2000; Herrera and Dudley 2003; Hardesty-Moore et al. 2020). However, its effects on microeukaryotes have not yet been investigated. Maceda-Veiga et al. (2016) found a positive correlation between *A. donax* invasion and the abundance of soil collembola, which are microbial grazers (Rusek 1998), as many microeukaryotes are (Finlay 1997). Thus, *A. donax* leaf litter may impact microeukaryotes, generating a trophic cascade that affects mosquito larval development (Fig. 1), but this question warrants detailed investigation.

Here, we investigated this question using leaf-litter microcosms spanning a biomass gradient of the invasive non-native *A. donax* relative to the native *P. australis*. Although both plant species have reported invasive populations around the world (Lambert et al. 2010; Meyerson et al. 2010), *P. australis* is the native plant most functionally similar to *A. donax* in Europe, including Spain, where our study was conducted (Jiménez-Ruiz et al. 2021). We compared their effects on water physicochemistry, microeukaryotic assemblages, and the larval development of *Culex pipiens* Linnaeus, 1758. The use of the common mosquito *Cx. pipiens* is relevant because it is native to Africa, Asia, and

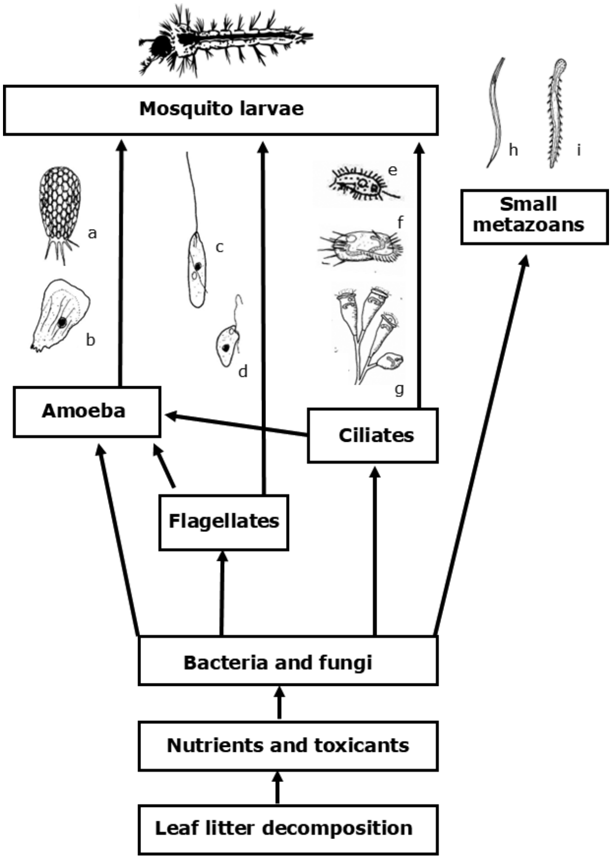

Figure 1. Schematic food web of the trophic cascade resulting from leaf litter decomposition and the interaction between microeukaryotes and mosquito larvae. Microeukaryotes can be prey, trophic competitors, and parasites and potentially alter the internal microbiota of mosquito larvae through changes in waterborne bacteria and fungi. Represented microeukaryotes are testate amoeba, **a**. Naked amoeba; **b**. Euglenid flagellate; **c**. Bodonid flagellate; **d**. Planktonic ciliate, **e**; crawling ciliates; **f**. Sessile ciliate; **g**. and micrometazoa (small metazoa): nematoda, **h**. and oligochaeta, **i**.

Europe, and adults can carry pathogens of human and veterinary importance, such as West Nile virus, St. Louis encephalitis virus, filariasis, and avian malaria (Becker et al. 2010; Brugueras et al. 2020). Our hypothesis was that the different releases of chemicals into water from *A. donax* and *P. australis* leaf litter decomposition, and the resulting effects on microeukaryotic assemblages, would impact mosquito larval development differently. Because previous work has shown *A. donax* tissues to be unpalatable and low in nutritional value for herbivores and detritivores (Going and Dudley 2008; Maceda-Veiga et al. 2016), we predicted that *P. australis* would shape the microeukaryotic food web that best supports mosquito larval development. Furthermore, we expected the effects of *A. donax* leaf litter to intensify with increased biomass. Finding this dose–response curve is important for the management of invasive non-native species when complete eradication is not feasible.

Methods

Experimental design and organism sources

Sympatric and recently fallen *A. donax* and *P. australis* leaf litter from the surface of the bankside of three channels in the Delta del Llobregat, NE Spain (41°18'N, 2°06'E), was collected in June 2022. The leaf litter was pooled by species, air-dried, fragmented with scissors, and stored in resealable bags until the experiment began at the University of Barcelona (UB). To prevent cross-contamination, the scissors were cleaned with 70% ethanol between plant species. All leaf fragments were morphologically similar, suggesting similar senescence, and those with signs of degradation (e.g., dark spots attributed to fungal growth) were discarded.

We assessed the multitrophic effects of the invasive non-native *A. donax* relative to our native control *P. australis* within 1-L glass microcosms. Each microcosm had 1 ± 0.2 g dry weight of total leaf litter, and there were five plant species combinations (A0-P100, A25-P75, A50-P50, A75-P25, and A100-P0) with ten microcosms each. Each combination is a biomass percentage of the two plant species (e.g., A25-P75 = 1 g of 25% *A. donax* and 75% *P. australis*), so, for simplicity, we refer to them hereafter as percentages of *A. donax* (A0, A25, A50, A75, and A100). The quantity of leaf litter in ecosystems is highly variable spatially and temporally (Elosegui and Sabater 2009), but these values are reported in breeding habitats of *Cx. pipiens* in our study area in the Delta del Llobregat and have been used in other experimental studies (Cano-Rocabayera 2019).

Each microcosm was filled with the weighed leaf litter, ultrapure distilled water, and a mixed inoculum of 100 mL of water from three nearby ponds at UB (41°23'N, 2°07'E) to establish a natural microbial community. Additionally, ten microcosms filled with ultrapure distilled water and the inoculum were set as negative controls, that is, without the leaves. The water sample for the inoculum was collected with 5-L sterilized pots at three points around each pond, including swimming organisms and organisms attached to the pond wall. The water samples from all three ponds were then pooled and sieved through a 50-µm mesh to prevent the accidental introduction of macroscopic metazoans (e.g., *Cloeon* mayfly). The inoculum was then immediately added to the microcosms in an alternating sequence across treatments to minimize priority effects. We are confident that this inoculum contains a representative microbial assemblage because the ponds had native riparian vegetation, they experience climate events similar to the Delta del Llobregat due to their proximity, and one of the ponds is visited by waterbirds, which are well-known microbial dispersers (Maguire 1963). Furthermore, many microeukaryotes are likely cosmopolitan (Finlay 2002; Finlay and Fenchel 2004). After 15 days from having set the microcosms, leaf leaching had occurred and the microbial community had developed (Kaufman and Walker 2006; Pelz-Stelinski et al. 2010), so we introduced mosquito larvae into all microcosms, including those used as negative controls. The eggs of *Cx. pipiens* were obtained from a wild culture kept for > 20 generations at the Baix Llobregat Mosquito Control Service in the Delta del Llobregat. Eggs from multiple rafts were hatched in a single tray filled with distilled water, and 20 individually collected 1st instar larvae were assigned to each microcosm utilizing a plastic Pasteur pipette in the sequence used for the microbial inoculum.

The experiment was conducted in a single run in the UB aquatic facility at 21.4 ± 1 °C and a 12h:12h day-night cycle. Microcosms were under diffuse light at 10 lux to simulate the shadow habitats in which mosquitoes often breed and to prevent potential photodegradation processes occurring in plant infusions (Skiff and Yee 2015). The experiment was run until all mosquito larvae either pupated or died after being fed exclusively with the microbial food web resultant from the leaf-litter decomposition process or the pond water inoculum. Microcosms were surveyed in two steps as described below. First, microeukaryote assemblages were characterized prior to mosquito larval addition to isolate the effects of leaf litter types on microeukaryotes. Second, microeukaryote assemblages and water physicochemistry were examined after mosquito pupation to explore the relative importance of environmental conditions and microeukaryotes for mosquito larval development. The water-only negative controls confirmed that mosquito larval survival was dependent on the resources provided by leaf litter decomposition, while the variation in mosquito larval development across leaf types identified the best culture conditions for mosquito larvae, allowing us to assess the ecological effects of different basal resources without the confounding effects of artificial nutrient supplements to mosquito larvae (e.g., yeast and fish food).

Microeukaryotic assemblages

Microeukaryotes were counted and identified in vivo, drop by drop, under a Leica® phase contrast light microscope at 200× magnification. To ensure data from this manual count were comparable, we screened five out of the 10 microcosms for each leaf litter combination and the negative controls so that the sampling times of all microcosms surveyed within each batch were comparable. There were two batches of sampling, one before adding mosquito larvae and one after mosquito pupation. Following the protocols of Maceda-Veiga et al. (2015, 2022), 25 mL of water was collected from the surface and 25 mL from the biofilm of each microcosm. Samples were obtained using a sterile 50 mL syringe (without a needle) moved in a zig-zag pattern to capture water just below the surface and directly from the leaf litter while scraping the biofilm. To prevent cross-contamination, the syringe was cleaned with 70% ethanol and rinsed with distilled water between samples.

Before analysis, each water sample was homogenized via ten manual inversions, and two 25 μL replicates were fully screened for microeukaryotes. This sampling volume follows the quantitative technique described by Madoni (1994) using 25 × 25 mm coverslips. This method ensures consistent water film thickness, facilitates drop-by-drop screening, and prevents sample desiccation during manual counts.

Furthermore, this volume is statistically sufficient to characterize the core communities of wastewater-treatment plants, which are typically more taxonomically diverse than those in our microcosms (e.g., Canals et al. 2018; Maceda-Veiga et al. 2015). The thin liquid layer under the coverslip standardizes the focal plane, facilitating the tracking and identification of fast-moving ciliates, such as *Cyclidium*, while preserving the characteristic swimming behaviors required for *in vivo* identification. While no method is perfect, this approach strikes a balance between high-precision *in vivo* identification by the same expert taxonomists and the need to process all replicates within comparable sampling times. To be confident, we also performed a second rapid screening at the end of each count to ensure that no congregated taxa at the edges or rare individuals were overlooked. Microeukaryote density was expressed as individuals per mL (ind/mL) (Madoni 1994; Canals et al. 2018).

To complement the results of the taxonomic approach, microeukaryotes were grouped into relevant functional identities based on habitat use, mobility, and diet, using published literature and our own observations and expertise. The functional guilds were amoeba, flagellates, crawling ciliates, planktonic ciliates, sessile ciliates, and worm-like micrometazoans. All were bacterivores except large amoebae and lecanid rotifers, which can prey upon other microeukaryotes (Rodríguez-Zaragoza 1994; Esteban and Fenchel 2020). Microeukaryotes also had distinct habitat preferences and motility patterns. Planktonic feeders (e.g., flagellates and planktonic ciliates) feed in the water column, while bottom feeders (e.g., amoeba, crawling ciliates, sessile ciliates, and micrometazoans) feed on the substrate or close to it, such as the stalked sessile ciliates (Curds and Cockburn 1970). Additionally, taxa exhibited different locomotion patterns, *i.e.*, pseudopodal movement (amoeba), flagellar movement (flagellates), ciliary movement (ciliates), and worm-like movement (micrometazoa), resulting in varying swimming velocities (ciliates > flagellates > amoebae and micrometazoa) (Esteban and Fenchel 2020). This classification is inherently linked to other biological traits, such as the shorter generation times of flagellates compared to ciliates and micrometazoa (Langlois et al. 2009). Consequently, changes in the density of these guilds can inform significant shifts in organic matter processing and potential differences in the vulnerability of microeukaryotes to mosquito larval predation.

Additionally, the density of fungal filaments was estimated in five microcosms per leaf litter treatment as described in Salvadó (2016). Briefly, we counted the intersection points of filaments under a Leica® light microscope after the same water sample was screened for the other groups of microeukaryotes. The counting was done using well-homogenized samples of 25 µL in 30 microscope fields at 100× magnification coupled with a video camera, and the density of fungal filaments was expressed as m/mL.

Mosquito larval development

Microcosms were examined daily for pupae, which were collected using a Pasteur pipette and individually placed in Eppendorf tubes at -20 °C until analysis. For each microcosm, we recorded the total number of larvae that reached the pupal stage (i.e., pupae number) and the total pupae mass. To do so, we thawed the pupae, removed excess water on a Kimwipe®, and weighed each pupa individually to the nearest ± 0.0001 g utilizing a Gram® electronic microbalance ER-182A. We also recorded the average number of days larvae needed to reach the pupal stage (i.e., mean larval developmental time) and the proportion of females by sexing pupae at x40 power magnification following the illustrations of the tenth abdominal segment by Moorefield (1951). We included female proportion as a response variable because females require a longer larval period and higher nutrient accumulation to achieve their larger body mass compared to males (Becker et al. 2010). Consequently, females may be more vulnerable to declining water quality or food resources than males.

Water physicochemical covariates

Water physicochemistry in microcosms was likely to have changed due to differences in leaf litter decomposition, organisms' metabolism, and mortality. To account for this variability when establishing associations between mosquito larvae and microeukaryotes, we measured general water chemistry, nutrients, and other essential and non-essential elements in each microcosm at the end of the experiment. pH, dissolved oxygen concentration and saturation (mg/L and %), and electrical conductivity (mS/cm) were measured using a digital probe (CRISON, Model: 507 and CRISON, Model: 524, Spain). All measurements were sampled from the center of the microcosms just below the surface, where *Cx. pipiens* larvae primarily spend their time breathing and/or feeding (Becker et al. 2010). In addition, a 75 mL water sample was filtered from each microcosm using a 0.70 μm glass microfiber filter and used for different analyses: (1) 25 mL was acidified with HCl prior to measuring dissolved organic carbon (DOC, mg/L) using a SHIMADZU TOC-V CSH instrument; (2) 25 mL was used to determine ammonium concentration (mg/L) using a SHIMADZU UV-1700 Pharma Spectrophotometer (Elosegui and Sabater 2009); and (3) 25 mL was sent to the UB Technical Scientific Services to measure potentially important cations released during leaf litter decomposition using inductively coupled plasma equipment (ICP-OES and ICP-MS). We measured major tissue constituents (e.g., Si, P, S, Mg, K, and Ca), key elements in physiological processes (e.g., Cu, Fe, Zn, Mn, Se, and Na), and potential environmental pollutants (e.g., Pb, Al, As, Cr, Ni, Li, Sb, and Sn) (see further details in Wood et al. 2012a; Wood et al. 2012b; Monroy et al. 2014; Rivera-Pérez et al. 2017; Filipiak and Filipiak 2022). Some essential elements (e.g., Cu, Zn, and P) also act as pollutants at high concentrations (Wood et al. 2012a; Wood et al. 2012b). To guarantee analytical accuracy, the official UB Technical Scientific Services used calibration curves, blanks, and quality standards for water samples (http://www.ccit.ub.edu/EN/tq01.html).

Data analyses

The distribution of all variables was visually inspected to detect data skewness and potential outliers and determine the most appropriate modelling distributions.

To determine the effects of % *A. donax* leaf litter relative to the native control *P. australis* on microeukaryotes prior to mosquito addition, we used taxonomic richness, total density, taxonomic composition, and functional guild structure as response variables. For taxonomic richness and total density, we used the R function *lm* on $\log_{10}$-transformed data. As microcosms with more individuals are likely to have more taxa detected by chance, the pattern for taxonomic richness was confirmed using the residuals of a linear regression between taxon richness and total density (Legendre and Legendre 2012). Residuals informed taxonomic richness after removing the effect of density. For taxonomic composition and functional guild structure, Permutational Analyses of Variance (PERMANOVA) were used with the R function *adonis2* (Oksanen et al. 2024). Bray-Curtis distance on square root-transformed abundances was used to deal with skewness, and % *A. donax* was included as a categorical factor (Legendre and Legendre 2012). If PERMANOVA was significant at $P \leq 0.05$, we used the R function *simper* (Oksanen et al. 2024) to identify the most characteristic taxa for each leaf litter combination. The R function *betadisper* was used to ensure that these outputs were driven by leaf litter type rather than by data dispersion. For the functional approach, we used non-parametric Kruskal-Wallis tests followed by Mann-Whitney tests as post hoc tests to compare differences in density of each functional guild among leaf litters. To confirm the effects of litter addition on microeukaryotes, we also compared taxonomic richness and total density among microcosms without leaf litter and those with 100% *A. donax* or 100% *P. australis* using Kruskal-Wallis tests followed by Mann-Whitney tests as post hoc comparisons. *P* values in multiple pairwise comparisons were corrected using the Benjamini and Hochberg procedure (1995).

To determine the effects of % *A. donax* leaf litter relative to the native control *P. australis* on mosquito larval development, we used pupae number, total pupae mass, mean developmental time, and female proportion as response variables. We explored relationships of % *A. donax* with the response variables by means of the R function *lm* on $\log_{10}$-transformed data, except for the proportion of females, which was modelled using the R function *betareg* (Ferrari and Cribari-Neto 2004). Then, the same R functions were employed to explore the relative effects of water physicochemistry and the functional guild structure of microeukaryotes on the four mosquito variables. To evaluate the relative effects of microeukaryotes and water chemistry on mosquito development, we conducted a subsample paired analysis. Because microeukaryote characterization was performed on a subset of five out of 10 microcosms per treatment, we restricted this specific analysis to those replicates where water chemistry, microeukaryotes, and mosquitoes were examined simultaneously. All predictors were standardized using the R command *scale*. The R function *principal* was used to summarize variation in water properties in a Principal Component Analysis (PCA) (Revelle 2024), and the resultant PC scores were used as predictors. To improve the ratio between predictors and observations and reduce potential redundancy, one variable of each highly correlated pair based on Spearman's rho |0.8| was excluded following Bandalos and Boehm-Kaufman (2009). To reduce data skewness, all water properties and fungal filaments were $\log_{10}$-transformed, except pH and oxygen saturation. The density of fungal filaments was included as another covariate given its density in microcosms sampled after mosquito larvae addition. We found the minimum adequate model based on likelihood-ratio tests at $P \leq 0.05$ and the lowest Akaike Information Criterion value using the R function *drop1* (Legendre and Legendre 2012). To account for potentially non-linear relationships, we compared linear and quadratic fits for each response variable.

All analyses were conducted using R (v4.3.2; R Core Team 2023). Overall model fit was determined by means of adjusted R^2 values, and model assumptions were checked using diagnostic plots of model residuals (Legendre and Legendre 2012).

Results

Effects of leaf litter type on microeukaryote assemblages

Prior to mosquito larval addition, 35 taxa were recorded across all treatments (500,440 ind/mL) spanning six functional guilds, except crawling ciliates (Suppl.

material 1: table S1). No yeasts or algae were detected. Fungal filaments were present in 1% of the samples and were excluded from the analysis. Microeukaryote assemblages were dominated in density by planktonic ciliates (67%), while sessile ciliates were rare (0.15%). Microcosms containing leaf litter, regardless of species, had significantly higher microeukaryote densities (mean ± SD = $1.96{\cdot}10^4$ ± $1.68{\cdot}10^4$ ind/mL) and taxon richness (10 ± 2) than litter-free controls (900 ± 722 ind/mL; 10 ± 2; all $P < 0.001$). Increased % *A. donax* leaf litter relative to *P. australis* significantly increased both total density of microeukaryotes ($R^2 = 0.58$; $F_{1,23} = 32.2$; $P < 0.001$) and taxon richness ($R^2 = 0.41$; $F_{1,23} = 17.7$; $P < 0.001$). The association with taxon richness remained significant even after controlling for differences in total density ($R^2 = 0.25$; $F_{1,23} = 7.69$; $P = 0.01$).

Increased % *A. donax* leaf litter relative to *P. australis* significantly shifted taxonomic composition (PERMANOVA, $R^2 = 0.51$; $F_{4,24} = 5.27$, $P = 0.001$). SIMPER analysis revealed that these differences were due to flagellates (*Peranema* sp.), rotifers (*Lecane inermis* and other unidentified lecanids), pelagic ciliates (*Cyclidium* sp., *Cinetochilum* sp., *C. margaritaceum*, *Dexiotricha granulosa*, and *Ctedoctema* sp.), and the amoeba *Euglypha* sp. Consequently, increased % *A. donax* leaf litter also significantly shifted functional guild structure (PERMANOVA, $R^2 = 0.66$; $F_{4,24} = 9.53$, $P = 0.001$), particularly for flagellates (KW = 14.53, df = 4, $P = 0.005$), micrometazoa (KW = 19.99, df = 4, $P < 0.001$), and planktonic ciliates (KW = 15.87, df = 4, $P = 0.003$) (Fig. 2). No significant variation was detected in the densities of amoebae (KW = 6.17, df = 4, $P = 0.18$) and sessile ciliates (KW = 2.96, df = 4, $P = 0.56$). The densities of the other three guilds significantly increased with % *A. donax* leaf litter and stabilized thereafter once *A. donax* reached 25% of the litter mixture (Fig. 2).

Effects of leaf litter type on mosquito larval development

All mosquito larvae died in the microcosms without leaf litter (negative controls), supporting the essential role of leaf litter in fueling the microbial food web. Increased % *A. donax* leaf litter relative to *P. australis* significantly affected pupae number ($R^2 = 0.44$; linear: t = 4.14, quadratic: t = –2.71; both $P < 0.01$), total pupae mass ($R^2 = 0.18$; linear t = 3.24; $P < 0.01$), and mean developmental time ($R^2 = 0.63$; linear: t = –8.16, quadratic: t = 6.75; both $P < 0.01$) (Fig. 3), while female proportion was unaffected ($P = 0.69$; Suppl. material 1: table S2, fig. S1). Microcosms without *A. donax* produced fewer and lighter pupae and exhibited longer developmental times than mixed or pure *A. donax* microcosms. Pupae mass increased progressively with *A. donax* leaf litter %, while the effects on pupae number and mean developmental time were pronounced after 25% *A. donax* and stabilized thereafter, similar to the microeukaryotic responses (Fig. 3).

Relationships among mosquito larval development, microeukaryotes, and water physicochemistry

Prior to PCA, to reduce excessive correlation among water properties, several ions (Li, Mg, Na, P, K, and S) were excluded due to high correlation with conductivity (all Spearman's rho ≥ 0.8). We also excluded Cr for its high correlation with Al (0.82) and Ni for its high correlation with Cu (0.75). The PCA built with the remainder showed that PC1 (26% variance) loaded mostly on conductivity, NH_4^+, Al, Cu, Zn, and Si, while PC2 (18%) primarily included As, Fe, and Pb (Table 1).

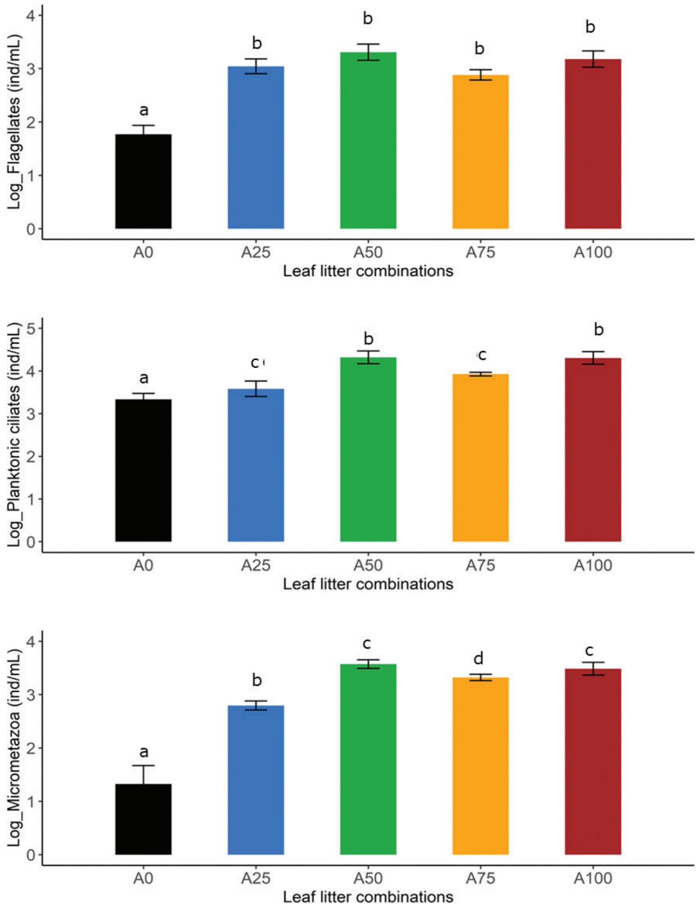

Figure 2. Changes in densities of the functional guilds of microeukaryotes (mean ± SD) significantly affected by *A. donax* leaf litter prior to mosquito addition. Response variables are plotted log10-transformed so that each unit change on the Y-axis represents a ten-fold increase in density. The letters on the top of the bars group treatments homogeneously at $P \geq 0.05$ based on Mann-Whitney tests corrected for multiple comparisons. Microcosms had five biomass percentages of leaf litter of the invasive non-native plant *Arundo donax* (A) relative to the native control *Phragmites australis* (P) (n = 10 replicates; A0 = 0% A + 100% P; A25 = 25% A + 75% P; A50 = 50% A + 50% P; A75 = 75% A + 25% P; and A100 = 100% A + 0% P).

Microcosms with 100% *P. australis* and *A. donax* separated clearly along PC1 (Fig. 4), indicating that *A. donax* leaf litter generally enriched water with many ions except Si compared to *P. australis* at similar leaf mass loss (±0.1 g; $P > 0.05$) (Suppl. material 1: table S3). PC2 showed a bell-shaped pattern, peaking at 25–50% *A. donax* with the levels of As, Fe, and Pb (Table 1).

Our modeling indicated that water physicochemistry (PC1 and PC2 scores) had a stronger influence on mosquito variables than fungal filaments or microeukaryotes quantified after mosquito pupation (Table 2). These patterns were apparent even when microcosms harbored a diverse assemblage of microeukaryotes, including a total of 50,160 microeukaryote individuals per mL distributed across all six functional guilds (Suppl. material 1: table S4). Flagellates dominated the assemblage, and micrometazoa were the rarest taxa recorded (Suppl. material 1: table S4).

Pupae number and mass increased with PC1 scores, while mean developmental time declined with both PC1 and PC2 scores (Table 2). Total pupae mass and female proportion were positively related to PC2 scores (Table 2). Microeukaryotes

Table 1. Loadings from a Principal Component Analysis (PCA) of the water physical and chemical properties measured in microcosms containing five biomass percentages of the invasive *Arundo donax* and the native *Phragmites australis* leaf litter. The weight of a variable is highlighted in bold when its loading was ≥ 0.7 in absolute value.

	PC1	PC2
pH	**0.72**	–0.26
Electrical conductivity	**0.80**	0.38
Dissolved O_2	0.30	–0.54
Dissolved organic carbon	–	0.21
Ammonium (NH_4^+)	0.58	–
Aluminum (Al)	0.62	0.32
Copper (Cu)	0.56	0.31
Selenium (Se)	0.46	–0.15
Manganese (Mn)	–0.19	0.54
Arsenic (As)	–	**0.70**
Tin (Sn)	0.55	—
Calcium (Ca)	0.38	0.56
Antimony (Sb)	–	0.55
Iron (Fe)	0.18	**0.74**
Zinc (Zn)	0.60	–
Silicon (Si)	**–0.78**	–
Lead (Pb)	0.51	0.67

Table 2. Results of the best models for explaining the variation in the four mosquito traits using water physicochemical properties, as informed by scores of PC1 and PC2, fungal filaments, and functional guilds of microeukaryotes as potential predictors. The models shown include only significant predictors based on likelihood-ratio tests at $P \le 0.05$.

Mosquito traits	Estimates	SE	*t-test*	*P* value
Pupae number (R^2 = 0.33)				
PC1 scores	0.14	0.05	2.67	0.01
Flagellate density	–0.11	0.05	–2.05	0.05
Total pupae mass (R^2 = 0.17)				
PC1 scores	0.01	0.004	3.00	0.006
PC2 scores	0.01	0.003	3.11	0.005
Flagellate density	–0.009	0.004	–2.09	0.04
Mean developmental time (R^2 = 0.69)				
PC1 scores	–0.12	0.02	–4.64	<0.001
PC2 scores	–0.07	0.02	–3.33	0.003
Female proportion (R^2 = 0.58)				
PC2 scores	0.03	0.007	3.58	0.001
Amoebae density	0.03	0.008	3.54	0.001

Estimates, standardized regression coefficients; SE, standard error; R^2, fit.

showed some weaker but significant associations. Pupae number and mass decreased with flagellate density, and female proportion was similarly associated with amoebae density and PC2 scores (Table 2). Fungal filaments, though more abundant with increased *A. donax* % (R^2 = 0.31; $F_{1,23}$ = 10.5; *P* = 0.003), did not significantly explain variation in any of the mosquito variables (Suppl. material 1: fig. S2).

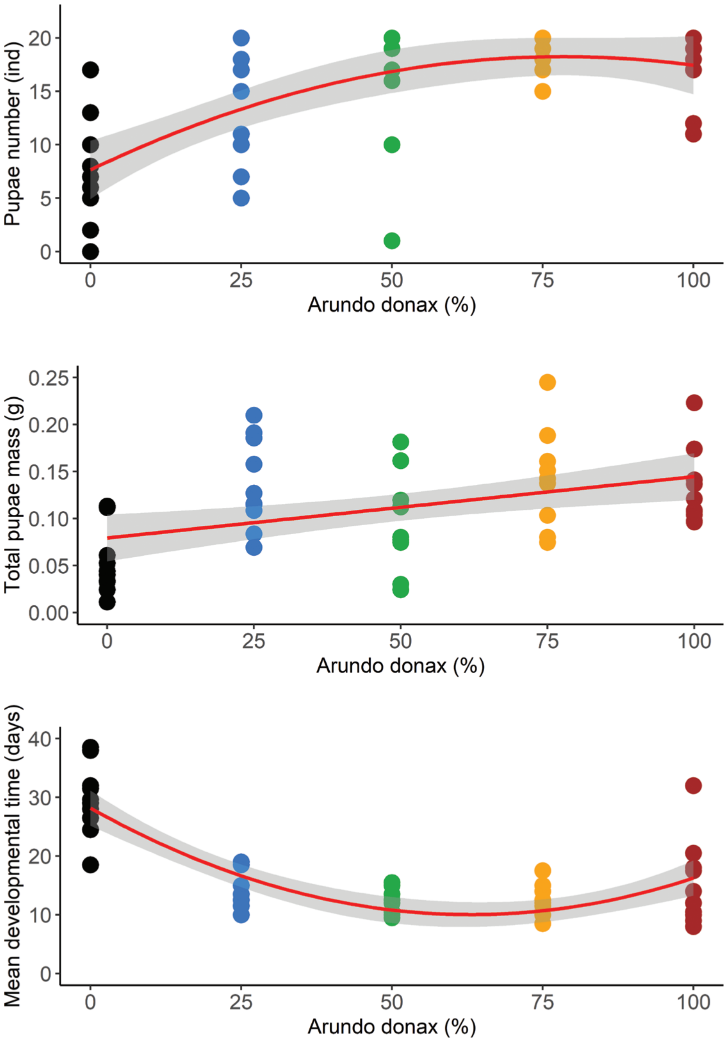

Figure 3. Fitted lines (± 95 CI) of the linear or quadratic responses of mosquito traits as a function of the five biomass percentages of leaf litter of the invasive non-native plant *A. donax* relative to the native control *P. australis* (n = 10 replicates shown as dots; A0 = 0% A + 100% P; A25 = 25% A + 75% P; A50 = 50% A + 50% P; A75 = 75% A + 25% P; and A100 = 100% A + 0% P).

Discussion

Our findings demonstrate that increased *A. donax* leaf litter % relative to the native control *P. australis* affects microeukaryote assemblages and alters the development of *Cx. pipiens* larvae as the apex consumer. Contrary to expectations, *A. donax* promoted mosquito larval development, and the biological responses often did not follow linear dose–response patterns.

Effects of leaf litter type on microeukaryote assemblages

Prior to mosquito larval addition, total microeukaryote density and taxon richness increased markedly in microcosms with leaf litter, confirming its key role in supporting freshwater microbial food webs (Ferreira et al. 2016; Reiss 2021). Specifically, flagellates, planktonic ciliates, and micrometazoa (mainly lecanid rotifers) reached the highest densities in *A. donax* microcosms, suggesting increased

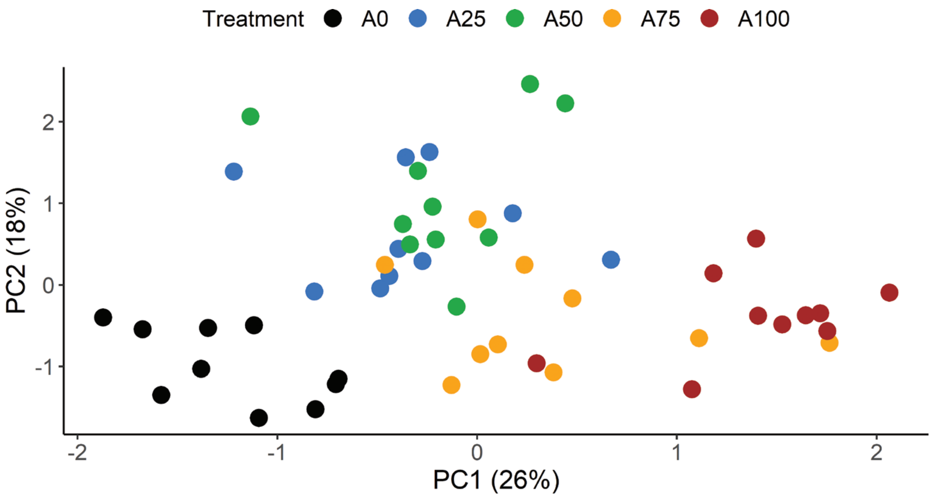

Figure 4. Principal component analysis (PCA) of the physicochemical water properties measured in microcosms containing five biomass percentages of leaf litter of the invasive non-native plant *Arundo donax* (A) relative to the native control *P. australis* (P) (n = 10 replicates shown as dots per leaf litter combination; A0 = 0% A + 100% P; A25 = 25% A + 75% P; A50 = 50% A + 50% P; A75 = 75% A + 25% P; and A100 = 100% A + 0% P). The percentage values (%) on each axis represent the amount of variance explained by each principal component (PC). To improve visualization of the PCA plot and interpretation of the effect of each water property on the ordination, the loadings of each water property are detailed in Table 1.

bacterial productivity driven by nutrient release from *A. donax* leaf litter, as these microeukaryotes are bacterivores (Curds and Cockburn 1970; Finlay 1997; Fiałkowska and Pajdak-Stós 2008). Rotifers, capable of feeding on larger microbes (Arndt 1993; Fiałkowska and Pajdak Stós 2008), also likely contributed to the reduced frequency of filamentous fungi seen in microcosms prior to mosquito larval addition (Suppl. material 1: table S1) compared to microcosms after mosquito pupation when rotifers were not present (Suppl. material 1: table S4).

This enhanced microbial activity with *A. donax* leaf litter supports a previous field study linking its presence to elevated densities of collembola (Maceda-Veiga et al. 2016), which are microbial grazers (Rusek 1998). However, a study reported that phytotoxins in *A. donax* tissues limit the activity of larger invertebrates feeding on the decomposed leaves (Going and Dudley 2008), suggesting that toxicity may depend on the animal studied. Differences may also reflect the decomposition stage and colonization history of the leaves by microeukaryotes. We did not track successional changes, but microeukaryote assemblages often reflect the environmental conditions of days and even weeks before sampling time (Curds and Cockburn 1970; Foissner 1999). Thus, *A. donax* leaf litter perhaps developed a more diverse assemblage of microeukaryotes than *P. australis*, in part due to distinct microbial communities colonizing leaves before submersion (Bamforth 1980; Esteban and Fenchel 2020), despite the two plant species growing in sympatry. While changes in individual taxa within the speciose microbial assemblages may seem of minor ecological importance, microeukaryotes often function as consortia of specific taxa to decompose substrates (Mboyi et al. 2017; Kachienga and Momba 2018). This may apply to the decomposition of *A. donax* leaf litter, but we feel that the study of microeukaryotes in invasion ecology is still too limited to answer this question definitively.

Effects of leaf litter type on mosquito larval development

Mosquito larval performance mirrored our microbial responses prior to mosquito larval addition. *Arundo donax* litter had more and larger pupae and shorter mean developmental times compared to *P. australis*. Larger pupae imply greater reproductive potential and longevity in mosquito females (Steinwascher 1982; Juliano et al. 2014), which, from a public health perspective, suggests that *A. donax* could increase *Cx. pipiens* abundance and biting nuisance (Farajollahi et al. 2011; Brugueras et al. 2020). Accelerated development also shortens the time window for mosquito control services to target mosquito larvae, the most efficient control procedure for managing mosquito populations (Becker et al. 2010). However, local mosquito populations depend on factors not addressed in this study, such as breeding site selection by mosquito females, including sites without leaf litter and with strong mosquito larval predation (Burkett-Cadena et al. 2013; Cano-Rocabayera et al. 2020). In any case, our finding that a 25% replacement of *P. australis* with *A. donax* significantly shifted larval traits adds further evidence for the designation of *A. donax* as one of the world's worst invasive plant species (Jiménez-Ruiz et al. 2021). Thus, promoting native vegetation that is less suitable for mosquito larvae could complement other integrated pest management strategies, including the use of *Bacillus thuringiensis* serovar *israelensis*, fish, and other predators such as odonates (Knight et al. 2003; Becker et al. 2010). A recent study also underscores the need to assess the effects of *A. donax* on consumers in combination with changing environmental conditions (Maceda-Veiga et al. 2025).

Relationships among mosquito larval development, microeukaryotes, and water physicochemistry

Model outputs revealed that water physicochemistry better explained mosquito performance than the microeukaryote densities recorded after mosquito pupation. Although correlations between PC scores and microeukaryotes were not a statistical issue, the microeukaryote densities were linked to water ionic composition (Spearman's $\varrho \leq 0.5$), consistent with their known bioindicator role (Curds and Cockburn 1970; Salcedo et al. 2024). Water chemical differences at the end of the experiment likely originated from leaf-derived decomposition, microbial metabolism, and differences in mosquito larval developmental time, as all organisms were ammoniotelic and contributed to water NH4+/NH3 levels (Wright 1995). Despite this, we posit that the plants, due to their comparatively greater biomass, were likely the primary drivers of water quality changes in microcosms through the leaf-litter decomposition process. Decomposition rates were not measured because the repeated sampling required would have altered microcosm habitat structure and the microeukaryote–mosquito food web, which was the focus of the study. In any case, *A. donax* microcosms exhibited higher nutrient enrichment (e.g., P, K, and S) despite similar initial litter masses, consistent with potential differences in decomposition rates between *A. donax* and *P. australis* (Jiang et al. 2023).

The abrupt responses in microeukaryote densities and mosquito larvae with *A. donax* leaf litter suggest that nutrients such as P, K, S, Na, and Mg—higher with *A. donax*—were limited in 100% *P. australis* microcosms. These elements are essential for physiological regulation in many animals, including mosquitoes (Dow 2017; Rivera-Pérez et al. 2017), and their addition may have stimulated both microbial growth and larval development (Kaufman and Walker 2006; Walker et al. 2010). Silicon in *P. australis* may have limited mosquito larval browsing, as reported for amphipod shredders (Schaller and Struyf 2013). Furthermore, although most elements remained within safe levels, Zn and Cu concentrations exceeded standards (5 µg/L and 30 µg/L, respectively), and non-essential As and Pb were detectable and are potentially toxic (Wood et al. 2012a, b), but mosquito larvae and microeukaryotes seemed to have tolerated these conditions well. A previous study reported that *P. australis* tissues store more metals than *A. donax* (Bonanno 2013), but this may not translate directly to our water measurements.

The significant effect of microeukaryotes on mosquito larvae may be explained by trophic competition, predation, intraguild interactions, and differences in the number of mosquitoes at the end of the experiment (Walker et al. 2010). The *Cx. pipiens* densities used were aligned with studies showing top-down effects on microeukaryotes (Kneitel and Chase 2004), though others report limited impacts (Skiff and Yee 2015). Furthermore, at post-pupation, microcosms contained up to 20 taxa, including known prey such as *Cyclidium* sp. (Suppl. material 1: table S4), setting the stage for potential predator–prey dynamics. In this regard, reduced flagellate density with increasing pupae could suggest that nanoflagellates, the dominant flagellate group (Suppl. material 1: table S4), fall within the prey size range of mosquito larvae but also that nanoflagellates compete with larvae for bacteria (Becker et al. 2010; Esteban and Fenchel 2020). The amoeba–female mosquito association posited additional complexity. Testate amoebae or their habitat may supply vital Si and Fe for insect cuticle synthesis (Richards 1951), yet the specific benefits for females is a key area of further investigation. Our study discards additional complexity due to the effects of protozoan epibionts such as *Epistylis* sp. and *Chilodonella uncinata* (Chapman 1974; Das 2003), as qualitative microscopic examination of 10 larvae from microcosms did not detect them. Future studies should determine if the observed shifts in microeukaryote assemblages alter internal mosquito microbiota, a key research area (Strand 2018).

Conclusion

Our study demonstrates that *A. donax* leaf litter promotes larval development in *Cx. pipiens*, a widely distributed mosquito species (Becker et al. 2010; Brugueras et al. 2020). Threshold responses observed between 0 and 25% *A. donax* leaf litter reveal high sensitivity of microbial food webs and mosquito larval development to even low invasion levels of *A. donax*. Consequently, managers should prioritize early removal of *A. donax* in invaded areas and restore these sites with native vegetation such as *P. australis*, though future studies should investigate mosquito breeding preferences in these habitats. Where eradication is unfeasible, it is necessary to examine how *A. donax* management can complement other mosquito control methods, such as the use of *B. thuringiensis* serovar *israelensis* and local predators (Becker et al. 2010). In this regard, our study highlights the role of water chemistry and microeukaryotes in explaining the trophic cascade generated by replacing *A. donax* with *P. australis*.

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Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

Use of AI

No use of AI was reported.

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Author contributions

OCR: Conceptualization, Investigation, Formal analysis, Resources, Writing – original draft, Writing – review and editing. YYB: Conceptualization, Data Curation, Formal analysis, Writing – original draft. HS: Investigation, Methodology, Writing – review and editing. MGB: ISS: Methodology, Writing – review and editing. CB: Writing – review and editing also Conceptualization. MV: Investigation, Writing – review and editing. FS: Investigation, Writing – review and editing. CA: Writing – review and editing. AMV: Conceptualization, Investigation, Funding acquisition, Methodology, Data Curation, Formal analysis, Resources, Writing – original draft, Writing - review and editing.

Author ORCIDs

Islam S. Sobhy https://orcid.org/0000-0003-4984-1823
Colin Berry https://orcid.org/0000-0002-9943-548X
Montserrat Vilà https://orcid.org/0000-0003-3171-8261
Alberto Maceda-Veiga https://orcid.org/0000-0002-1736-4631

Data availability

All of the data that support the findings of this study are available in the main text or Supplementary Information.

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Supplementary material 1

Additional figures and tables

Authors: Oriol Cano-Rocabayera, Ydsell Y. Bonilla, Humbert Salvadó, Marc Garcia-Bargalló, Islam S. Sobhy, Colin Berry, Montserrat Vilà, Francesc Sabater, Carles Aranda, Alberto Maceda-Veiga

Data type: pdf

Explanation note: **table S1.** Descriptive statistics (mean, standard deviation, and minimum–maximum range) of all microeukaryotes recorded in microcosms containing leaf litter of the invasive plant *Arundo donax* relative to the native *Phragmites australis* prior to mosquito larval addition. **table S2.** Results from linear or quadratic models explaining variation in the four mosquito traits as a function of the five biomass percentages of leaf litter from *A. donax* relative to *P. australis*. **table S3.** Mean and standard deviation (SD) of all water physicochemical properties measured in microcosms containing leaf litter of the invasive plant *Arundo donax* relative to the native *Phragmites australis*. **table S4.** Mean and standard deviation (SD) of all microeukaryotes recorded in microcosms containing leaf litter of the invasive plant *Arundo donax* relative to the native *Phragmites australis*. **fig. S1.** Boxplots showing differences in female proportions among leaf litter biomass percentages of *Arundo donax* (A) and *Phragmites australis* (P). **fig. S2.** Fitted lines (± 95 CI) of the responses of fungal filament density (m/mL) as a function of the leaf litter biomass of *Arundo donax* relative to that of the native *P. australis*.

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