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Diatom flora in Mediterranean intermittent streams: serious threat for endangered species

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ABSTRACT

In the context of global environmental changes, Mediterranean rivers are considered highly endangered. Temporal and spatial increases of the dry stretches during the summer lead to the loss of river tridimensional connectivity, which represents a major threat for freshwater biodiversity.

In this study, we aimed at exploring the response of diatom communities to summer droughts by analyzing taxonomical composition, specific ecological requirements, ecological guilds and percentages of endangered species. The evolution of diatom communities was monitored under both intermediate and intermittent flows, with traditional and innovative sampling procedures, i.e. collecting diatoms from transects and microhabitats, respectively. Microhabitats differed in terms of water velocity, substrate, isolation and presence of macrophytes.

Diatom flora was mainly composed of β-mesasoprobous taxa. We highlighted an increase of species considered as aerophilous and planktonic in sites characterized by intermittent flow. In general, ecological guilds did not respond to hydrological disturbance as expected. Statistical models identified the maintenance of a minimum of 0.20 m/s flow velocity as the main factor influencing the abundance of endangered species. Conversely, flow instability, lentification and habitat fragmentation represented the major threats for endangered species.

In conclusion, diatoms can provide useful information to improve river management practices when faced with an increasing water scarcity scenario. Water stability and river habitat heterogeneity strongly favor the presence of endangered diatom species. In the absence of these conditions, isolated pools surrounded by dry riverbed are very important habitats to be preserved, representing the only *refugia* for benthic diatom communities during summer.

Keywords: Red List, Bacillariophyceae, hydrological instability, pools

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INTRODUCTION

The Mediterranean basin is considered one of the most important biodiversity hotspots worldwide in terms of endemic species, and is considered to be greatly under threat (Cuttelod et al. 2008; Myers et al. 2000). Within this setting, Mediterranean freshwater ecosystems are considered highly endangered, more so than terrestrial ones (Sala et al. 2000), with a potentially huge loss of biodiversity. According to the review published by Dudgeon et al. (2006), the major threats for freshwater biodiversity can be both local (i.e. overexploitation; water pollution; flow modification; destruction and degradation of habitat; exotic species invasion) and global (environmental changes, such as nitrogen deposition; global warming; shifts in precipitation and runoff patterns). Biodiversity conservation and habitat integrity in rivers can be partly guaranteed by maintaining the natural flow stability (Dudgeon et al. 2006). With respect to diatoms, their diversity and species richness in Mediterranean streams are more closely related to hydrological variables than to physical-chemical features (Ros et al. 2009). Mediterranean rivers are naturally characterized by hydrological variations with extreme flows during autumn and winter, with droughts in summer (Pardo and Álvarez 2006). This phenomenon, recently exacerbated by human impact, has led to a temporal and spatial increase of the dry stretches, especially during the summer months. The main consequences of this phenomenon are habitat fragmentation and loss of river tridimensional connectivity (longitudinal, transversal and vertical; Bonada et al. 2006). In this context, residual isolated pools play an essential role for benthic communities in terms of species survival (Ros et al. 2009), and significantly contribute to the recolonization of the stretches after the return of the water (Robson and Matthews 2004).

In general, diatoms have developed coping mechanisms in order to confront harsh conditions, and are able to produce different resting forms, namely resting cells, spores and winter stages (McQuoid and Hobson 1996). Spore production requires a vast amount of energy, and cannot be considered, therefore, as a sustainable mechanism to face short-term environmental changes (Round et al. 1990), such as summer droughts in Mediterranean streams. Conversely, the production of resting cells is faster and uses less energy, as no additional silica is required. It has been recently demonstrated that *freshwater diatoms* (as defined in the classification of van Dam et al. 1994) are not able to survive desiccation as vegetative or resistance cells; on the contrary, *terrestrial diatoms* are able to face desiccation as resistance cells and, in some strains, as vegetative cells (Souffreau et al. 2013). In the same study, the authors demonstrated that acclimatization increases the tolerance of diatom strains to desiccation; this demonstrates that droughts can have a stronger negative impact on diatom communities in recently intermittent rivers than in Mediterranean regions.

An overall assessment of the diatom biodiversity in Mediterranean rivers is affected by many limitations such as the inconsistent application of taxonomical rules, the lack of historical data and the patchiness of the investigated areas (Tierno de Figueroa et al. 2013). Considering the big void in the literature, the role of the recent studies, which were mainly carried out in Spain (Boix et al. 2010; Ros et al. 2009) and in Portugal (Elias et al. 2015; Novais et al. 2014), is very important. In the scheme of the Water Framework Directive (WFD; European Commission 2000), an important step was carried out by Feio et al. (2014) who defined the Least Disturbed Conditions (LDC) for European Mediterranean rivers. In this context, defining the conservation status of diatom species is an important challenge. Currently, there is only one published Red List, compiled by Lange-Bertalot and Steindorf (1996) for German watercourses. This topic has been widely investigated in several studies carried out in the Alps, where the presence of rare and endangered taxa was shown to be correlated with habitat peculiarity. For instance, almost 50% of the diatom species recorded in the springs of the Adamello-Brenta Nature Park can be considered rare or threatened (Cantonati 1998). In the same way, about 30% of the taxa recorded in lentic habitats of the Maritime Alps Natural Park (mainly springs and peatbogs) can be considered "decreasing" or "endangered" (Falasco and Bona 2011). Conversely, the conservation status of diatom flora in Mediterranean streams was only recently explored by Novais et al. (2014), who highlighted a high proportion of endangered species in permanent and temporary rivers in Portugal and stressed the need to update and complete the diatom Red List with recently described taxa. As indicated by Denys (2000), the abundance of threatened species, as opposed to the number of species itself, can be considered a useful tool for assessing the loss of microhabitat and for evaluating possible deviation from pristine conditions. Therefore, the proposal of creating Red Lists on a scale of more ecologically homogeneous regions, such as hydroecoregions, should be seriously taken in consideration.

Given the lack of knowledge on this topic, we focused on the biodiversity status and presence of endangered diatom species in Mediterranean rivers in the Italian peninsula. The aims of our research were i) to investigate the diatom communities in Mediterranean streams in order to provide a baseline knowledge of the flora from both a taxonomical and an ecological point of view; ii) to evaluate diatom biodiversity and the presence of threatened and endangered species under stable (SPRING) and unstable (SUMMER) hydrological conditions.

Smucker and Vis (2011) highlighted a significant underestimation of diatom diversity when exclusively collected from epilithic habitats for documenting species distribution and for conservation purposes. Starting from this consideration, here we collected diatoms following two different sampling techniques, namely from transects located in riffles (T) and from microhabitats (MH). These two approaches were chosen in order to obtain the highest diatom diversity for each site; in this way, we were able to gather a significant environmental dataset that was used to better define the ecological preferences of endangered species and, at the same time, to evaluate the effect of habitat heterogeneity and fragmentation on diatom diversity via statistical models. In view of the results obtained, possible methods of management to mitigate the impact of drought are discussed.

MATERIALS AND METHODS

SAMPLING DESIGN

A total of ten Mediterranean streams in Liguria (NW Italy; Fig. 1 and Online resources 1), two in the Apennines N hydro-ecoregion (HER 64) and eight in the Ligurian Alps (HER 122) were selected. To reduce the environmental variability between sites, we only selected stretches characterized by a low anthropogenic pressure. For this purpose, we performed a land use analysis, and chose sampling sites with less than 50% of urban land use calculated in a 100 m buffer (Online resources 1). In addition, we checked historical physical-chemical data provided by the Ligurian Environmental Agency (ARPAL) and we carried out an *in situ* visual characterization of the sampling sites.

Five sampling surveys were carried out. The first one was conducted during SPRING (April 2014), under intermediate flow conditions, and involved all ten rivers. This sampling provided a baseline for the knowledge of the diatom flora in the absence of hydrologic disturbance. The other samplings were conducted during SUMMER (late June, July, August and September), when water scarcity characterized part of the stretches to different extents. In these sampling sessions, only five streams out of the initial ten were monitored, namely Argentina, Impero, Merula, Quiliano and Vallecrosia. In order to gain a better knowledge of diatom flora under extreme drought conditions, we decided to focus only on these streams, which have shown the most intermittent character in recent years (historical data provided by the Ligurian Environmental Agency). For each stream, we selected two sampling sites: the first one characterized by permanent flow throughout the year (upstream=UP), and the second one by intermittent flow, with part of the riverbed completely dry during summer (downstream=DW).

PHYSICAL-CHEMICAL PARAMETERS

In each sampling site, we measured: a) the main physical-chemical parameters, i.e. dissolved oxygen (DO) in the water, pH, temperature (TEMP), and conductivity (COND), using a multiparametric probe (Hydrolab mod. Quanta); b) total suspended sediments (TSS) following the Italian standard methods (APAT-IRSA. CNR, 2003); c) flow velocity (VEL), with a current meter (Mod RHCM Idromar) positioned at 0.05 m from the bottom of the riverbed; d) water depth (DEPTH) with a meter tape; e) soluble reactive phosphorous (SRP) and nitrate ($N\text{-}NO_3$) with a LASA 100 spectrophotometer, according to APAT-IRSA CNR standard methods (2003). Environmental features were evaluated *in situ* by visually attributing percentages to: the main substrate composition, macrophytes and algae coverage and checking the presence or absence of shade and connection with the main flow.

DIATOM ANALYSIS

In each site, six epilithic diatom samples were collected and kept separate for the analysis of diatom communities. We followed two different sampling approaches: one sample was collected in accordance with the *transect approach* (T), while the other five samples were collected using the *microhabitat approach* (MH). The T approach followed the standard procedure defined by the European Committee for Standardization (2003). We chose at least five cobbles from the main flow and we collected periphyton by scraping their upper surface using a toothbrush. Considering the MH approach, five microhabitats were selected at each site. Microhabitats were differentiated in terms of current velocity, depth, dominant substrate, presence of macrophytes and shade. When present, isolated pools were preferentially selected. In both cases, we chose to sample only cobbles, in order to reduce the effect of the substrate typology and focus on the influence of the surrounding microhabitat. All diatom samples were preserved in ethanol. Samples were subsequently treated in the laboratory following the standardized method (European Committee for Standardization 2003) by cleaning them with hydrogen peroxide (30%) and HCl. Slides for observation at the light microscope were mounted by means of Naphrax®. Diatom identification was based on several diatom floras and monographies, as well as on recent taxonomic papers (Bey and Ector 2013; Blanco et al. 2010; Ector et al. 2015; Falasco et al. 2013; Hofmann et al. 2011; Krammer 1997 a, b, 2002, 2003; Krammer and Lange-Bertalot 1986-1991 a, b; Lange-Bertalot 2001; Lange-Bertalot and Metzeltin 1996; Reichardt 1999; Werum and Lange-Bertalot 2004), and at least 400 valves per sample were identified. Diatom communities were analyzed in terms of biodiversity, taxonomical and functional composition of the communities and presence and relative abundance of endangered taxa. The recorded species were classified by means of the OMNIDIA 5.3 software with 2015 database, on the basis of their ecological preferences, habitat (Denys 1991), moisture, pH and trophic state (van Dam et al., 1994), and conservation status (Lange-Bertalot and Steindorf 1996).

The *Correspondence Analysis* (CA), which is an unconstrained multivariate technique, was applied to the community data in order to visualize the dissimilarities of samples in terms of species composition. Data from SPRING and SUMMER were kept separate. Data in the species matrices were first square root-transformed to achieve a normal distribution. For this analysis, data from the samples collected with the MH approach were merged together. A total of 40 samples including 98 species, and 66 samples including 121 species were used for the SPRING and the SUMMER seasons, respectively.

In order to understand if there were endangered taxa typical of specific habitats, we performed the Indicator Species Analysis (ISA) on samples collected from all the sampling operations against the following groups: months, rivers, sampling site location, sampling methods, flow velocity, water depth, shade, isolation, dominant substrate, macrophyte presence, algae presence (see Table 3 for further details on group definition).

STATISTICAL MODELS

To determine which environmental parameters may favor the presence and abundance of endangered species, we applied Generalized Linear Mixed Models (GLMMs) assuming a Poisson error distribution (Zuur et al. 2009). Two different model structures were tested; the first was the *mesohabitat model*, including month, sampling site, sampling approach and disturbance as fixed effects. We considered samples collected under flow instability (i.e. MH samples from DW during SUMMER) as being disturbed, while all the remaining samples were considered as being collected from undisturbed conditions. The second model was the *microhabitat model*, including flow velocity (categorical variable: group 0 = $v \leq 0.20$ m/s; group 1 = $v > 0.20$ m/s), water depth and macrophyte coverage as fixed effects. We expressed flow velocity as a categorical variable because of the high imbalance towards zero values. Given the spatial dependence of the data, we applied the mixed procedure to include two grouping variables (river and site) as random factors, in order to account for the variation that they introduce into our samples. Before performing GLMMs, data were firstly explored via boxplots to assess the presence of extreme values and avoid unusual observations that may influence the estimated parameters (Zuur et al. 2009). CA was performed with the package *vegan* (Oksanen et al. 2015) and the ISA was performed with the package *indicspecies* (De Caceres and Legendre 2009) in R environment (R Core Team 2015), while GLMMs were performed via the PROC GLIMMIX (SAS software 9.2).

RESULTS

PHYSICAL-CHEMICAL PARAMETERS

Physical-chemical parameters detected during the samplings are shown in Table 1 and in the Online Resource 1. Environmental parameters were comparable between the two sampling seasons, with the exception of minimum-recorded values of DO, which were lower in SUMMER than in SPRING. In UPs, the lowest DO values (27.4%) were reached in the Argentina river, in a shaded lateral pool characterized by silt and coarse particulate organic matter (CPOM) as the main substrates. In DWs, the lowest DO values were generally associated with isolated pools, reaching extreme values of 15% and 27%. Nutrient concentrations were low in most of the studied stretches, with SRP levels being contained within the highest quality class in all cases, and nitrates within the second class (Italian Water Legislation D. Leg. 152/2006 and successive ones) in both the intermediate and low flow periods. In accordance with this consideration, chemical parameters were generally below the thresholds proposed by Feio et al. (2014) for the definition of the LDC for European Mediterranean rivers, with the exception of some values for nitrates in DW sites in SPRING and of DO concentration in SUMMER. TSS values were moderate; the highest value (21.17 mg/l) was observed in Varatella (DW), probably due to the presence of outfalls. The pH ranged from circumneutral to alkaline values and reflected the geology that mainly consists of limestone, sedimentary rocks and ophiolites, which dominates the Western part of the region. Conductivity decreased from West to East, following the gradual change in the geological composition.

DIATOM ANALYSIS

Biodiversity

The complete checklist of all the taxa detected in the samples, their ecological requirements, life forms, ecological guilds and conservation status are displayed in the Online Resource 2. A total of 126 diatom samples were analyzed for the SPRING season and a total of 171 taxa belonging to 44 genera were identified. On average, the number of species that composed the communities was comparable in the UP and DW sites (Table 1), as well as the Shannon diversity index, with highest median values in the MH samples of the UPs (S=2.72).

A total of 240 diatom samples were analyzed for the SUMMER season and a total of 241 taxa belonging to 58 genera were identified. In these samples, species richness was, on average, higher in the UPs than in DWs (Table 1). Regarding biodiversity, Shannon values were higher in the UP than DW sites in June and July, while no substantial differences were observed in August and September, when the median values were comparable. In UPs, MH samples hosted higher biodiversity in June and July (H_{MEDIAN}: June = 3.18; July = 3.25) than T (H_{MEDIAN}: June = 3.02; July = 3.19). Conversely, the MH samples generally showed a negative effect on diatom biodiversity in the sites most subjected to hydrological disturbance i.e. DWs (H_{MEDIAN}: June = 2.60; July = 2.64; August = 3.08). Despite this, we observed some outliers of the Shannon index in DW isolated pools during August (H_{MAX} = 4.07), similar to the results obtained by Ros et al. (2009).

Community composition

In all samples, *Achnanthidium minutissimum* was the most abundant and frequent species, followed by *Achnanthidium pyrenaicum.* In the Argentina stream, *Achnanthidium delmontii* was also consistently present in both UPs and DWs, with mean relative abundances of 24.4% and 15.3% in T samples, respectively. In the Arrestra stream, in addition to *A. minutissimum* and *A. pyrenaicum,* the DW site was characterized by the presence of *Diatoma ehrenbergii* (mean relative abundance of 27.0% in the T sample). Communities in the Impero stream were dominated by *A minutissimum,* but *Amphora pediculus* also presented high values of relative abundance, especially in the UPs. The Merula stream presented the most unusual flora, namely *Encyonopsis sumbinuta* and *E. minuta* were found in both UPs and DWs, as well as *Cymbella subtruncata;* in DWs, *Denticula kuetzingii* was often present with an average relative abundance of 10.1% in T samples. The genus *Encyonopsis* was also abundant in the Vallecrosia stream, in particular *E. minuta* and *E. subminuta*. In the Porra stream, the evenness was higher in UPs than DWs; as well as *A minutissimum,* communities were composed of *Nitzschia inconspicua*. In the UPs, we mainly found sensitive species such as *Cocconeis lineata* and *Achnanthidium subatomus*, while in the DWs a large proportion of the community was represented by the tolerant species *Mayamaea permitis*. In the Sansobbia stream, 11% of the UP community was characterized by *Nitzschia fonticola*, while *Encyonema silesiacum* represented 11% of the DW community. In the Varatella stream, *N. fonticola* also presented high values of relative abundance (22.5%) in UPs, while *Achnanthidium lineare* characterized the DW station (15.0%). In the Quiliano stream, the genus *Fragilaria* (and in particular *F. rumpens*) was frequently recorded, especially in the DW station, where the presence of *Cymbella tropica* was also important. In the Sciusa stream, the genus *Gomphonema* was highly represented in the UP station, while *Cymbella excisa* was abundant in DWs.

The CA performed on the SPRING biological dataset (Fig. 2a) revealed a strong dissimilarity between samples collected in different rivers. Moreover, sites located on the negative part of the CA2 axis belonged to streams with higher values of conductivity (>400 µS/cm). Given these results, the importance of mineral content on diatom assemblages collected from Mediterranean streams with comparable nutrient levels was once again confirmed (Sabater et al., 1988; Blanco et al., 2008).

Considering the CA performed on the SUMMER biological dataset (Fig. 2b), the site separation driven by stream identity was even more evident, highlighting the peculiarity of the diatom flora in Mediterranean streams.

Ecological requirements

In SPRING, diatom communities were mostly composed of β-mesosaprobous species (60% of the total abundance; van Dam et al. 1994), confirming the good water quality revealed from the chemical analyses. In general, 83% of the detected species preferred mean values of salinity and only 9% can be considered as brackish-freshwater taxa (i.e. 500-1000 mg Cl^-/l or 0.9-1.8 % of salinity; van Dam et al. 1994). The most abundant species belonging to this category were *Navicula gregaria* and *Nitzschia inconspicua*. Concerning moisture requirements, 31.6% of the recorded species were classified as mainly occurring in water bodies, but also regularly present on wet and moist places (MOIST=3); while 3.5% were classified as mainly occurring in wet and moist or temporarily dry places (MOIST= 4; van Dam et al. 1994).These taxa were *Adlafia minuscula* and *Geissleria acceptata*. According to the classification of Denys (1991), *A. minutissimum*, one of the most abundant species recorded in this study, should also be considered as commonly recorded in dry subaerial habitats. No strictly terrestrial species were detected. In terms of current velocity, most species (70%) were indifferent to water flow (Denys 1991).

There were differences observed in terms of ecological requirements for SUMMER species. In June, diatom communities were mainly composed of β-mesosaprobous taxa. *Achnanthidium minutissimum* and *A. pyrenaicum* dominated the communities, with 70% of total relative abundance in both UP and DW, with no differences between MH and T samples.

We observed a higher abundance of species belonging to the genus *Cocconeis* in the UP sites, probably due to a greater presence of aquatic macrophytes. In the DW sites (in particular in MHs), we noted a higher relative abundance of *Denticula kuetzingii* and *Fragilaria pararumpens*, as well as of taxa belonging to the genus *Cymbella*. In July, the growing presence in the Argentina river of *Achnanthidium delmontii* was evident, known for being an invasive species, in accordance with the criteria proposed by Coste and Ector (2000), along with a higher relative abundance of *Amphora pediculus* in the UPs. During this sampling session, *D. kuetzingii* was no longer exclusive to the DW sites, but was also recorded in the MH samples of the UP sites. In August, the abundance of *A. minutissimum* was drastically reduced, especially in the UPs, to the same levels as *A. pyrenaicum,* which was almost not recorded in the DWs. There was, however, a general increase of more tolerant species, considered as α-meso-polysaprobous, such as *Eolimna minima*, *Gomphonema parvulum* and *Ulnaria ulna*. In the UP sites, the second most abundant species was *A. pediculus*; moreover, the abundance of *Achnanthidium delmontii* doubled in comparison with the previous sampling session. There was a general increase in the relative abundance of species of the genus *Encyonopsis*, namely *Encyonopsis minuta* and *E. subminuta*, compared to the sampling in July. In September, species compositions were similar to those found in August, with slightly lower abundance of α-meso-polysaprobous taxa.

Concerning moisture requirements, 30.6% of the species recorded in SUMMER were classified as "MOIST=3". Compared to April, we observed an increase in the number of species classified as mainly occurring in wet and moist or temporarily dry places (MOIST=4; van Dam et al. 1994), representing 4.4% of the total species. Within this category, *Fragilaria alpestris* and *Halamphora montana* were the most frequent and abundant, despite only being found as a few individuals. Only one strictly terrestrial diatom was recorded, namely *Adlafia bryophila*, found in the Vallecrosia UP site, in a slightly shaded MH, characterized by slow flow and 100% filamentous and mat algal riverbed coverage. Species belonging to the MOIST categories 3 and 4 were almost exclusive from the MH samples, but no differences among sampling months were observed.

Concerning flow velocity, most of the species were indifferent to water speed. However, we detected three limnophilous taxa, namely *Amphipleura pellucida*, *Cymbella neoleptoceros* and *Diploneis elliptica*. In particular, *C. neoleptoceros* presented the highest percentages in the Impero DW site during September, when the hydrological disturbance was at its maximum.

Considering functional traits (Table 1), during SUMMER, colonial taxa were more abundant in the DW sites, where they represented, on average, more than 10% of the communities. No substantial differences were observed between the T and MH samples. This result confirmed the preference of colony-forming diatoms for lentic habitats (Rimet and Bouchez 2012) and for unpredictable water flow (Passy 2002). Contrarily to our expectations, *low profile* guild was generally more abundant in the UP than DW sites, while the *high profile* guild was much more abundant in the DW sites. As also observed by Elias et al. (2015), in our research the physical disturbance created by the drought did not increase the relative abundance of the *motile* guild, as we would have expected. Indeed, the *motile* guild was generally more abundant in the UP sites, and more abundant in the MH than in T samples, with the exception of the UP sites in September. Species considered as *adnate* were much more abundant in the UP sites where they preferred the MHs. We observed an opposite trend for the *peduncolate* taxa (both stalked and pad-attached to substrate), which presented a preference for the DW sites. For both UP and DW sites, *peduncolate* taxa were more abundant in the T than MH samples with the exception of DWs in September. The highest peaks in abundance for taxa forming mucous tube colonies were found in the DW sites during the hottest months and generally in the MH samples.

Conservation status: Red List species

The percentage of recorded species belonging to different conservation categories is summarized in Table 2. The number of species considered as being endangered per sample was higher in SPRING than in SUMMER, in particular for taxa classified as threatened with extinction. From the results, it is important to note that more than 30% of the recorded species in both SPRING and SUMMER were still not classified in accordance with the Red List. Indications on the statistically significant occurrence of taxa in terms of months, rivers, site location and sampling methods, water velocity and depth, shade, isolation, dominant substrate, macrophyte and algae coverage are shown in Table 3. Throughout the entire the sampling period, we recorded *Didymosphenia geminata* among the "threatened with extinction" taxa. *D. geminata* showed a preference for sites in which macrophytes consistently cover the riverbed (ISA; p= 0.023). Of the "endangered" species, *Achnanthidium lineare* and *A. gracillimum* were the most abundant and frequent in SPRING, and were mainly present in the Arrestra stream, characterized by high habitat integrity, with peaks in abundance in the MH samples. In particular, a semi-isolated shallow pool and a deeper pool with abundant CPOM sheltered these two species, along with *Achnanthidium exile* that is considered as "decreasing". During SUMMER, *A. lineare* and *A. gracillimum* were again the most abundant species, with peaks during August and September. In particular, *A. lineare* represented more than 50% of the communities in three samples collected in the UP site of the Quiliano stream, during both August and September. All the samples were collected in shallow pools (flow velocity = 0 m/s and depth < 20cm) shaded by the riparian vegetation. *A. lineare* appeared to be limited by the presence of other benthic algal groups (ISA; p= 0.005) and preferred naturally shaded (ISA; p= 0.022) sites. *A. gracillimum* was found in the DW site of the Quiliano stream during September, and was present in all the samples (T and MHs) with the exception of the only isolated pool that was sampled. *A. gracillimum* was recorded in shallow (ISA; p=0.032) standing or flowing waters, always connected to the main flow and showed a statistically significant preference for microlithal as the main substrate (ISA; p=0.006). Under the same category, *Nitzschia gessneri* was present in the samples of June and July, with a clear preference for the MH samples. This species, not recorded in SPRING, reached peaks in the Merula river, in both the UP and DW sites. In particular, we recorded its presence in an isolated pool with intermittent water presence. *Navicula novaesiberica*, considered as “rare”, was abundant in the Varatella DW site, in a very shallow pool with standing water and high siltation. Among the “probably endangered” taxa, we highlighted *Ulnaria biceps* and *Gomphonema tergestinum* as the most abundant. The former presented the highest abundance during July and September at the DW sites of Vallecrosia (T) and Quiliano (MH) streams, respectively. These samples were collected in shallow (depth ca. 12 cm) flowing (velocity ca. 0.20 m/s) waters with a significant coverage of macroscopic filamentous green algae (from 60 to 100%). The populations were always composed of a few individuals, confirming the observations of Bey and Ector (2013). In the “decreasing” category, we detected 14 species, the most abundant of which were *Gomphonema lateripunctatum* and *Nitzschia tabellaria* that presented peaks in abundance during the warmer months. In particular, the Merula DW site hosted the highest abundance of *G. lateripunctatum* in July and September. During July, the species showed peaks in abundance in a pool connected with the main watercourse, presenting standing water and 35 cm of water depth. In September, *G. lateripunctatum* was found in the same stretch as in July, but with a peak in abundance in a MH with flowing water (velocity = 0.13 m/s and depth = 11 cm). The preference of *G. lateripunctatum* in the Merula stream can be explained by the fact that it is commonly found in the Mediterranean hydroecoregions with preferences for calcareous streams (Delgado et al. 2013; Gomà et al. 2004). This species was significantly present in pristine sites characterized by microlithal as the main substrate, and its abundance was not limited by isolation from the main flow. *Nitzschia tabellaria*, considered as being characteristic of habitats of high conservation value (Potapova and Charles 2007; Smucker and Vis 2011) was particularly abundant in the UP site of the Argentina stream.

STATISTICAL MODELS

Results of the statistical models showed that environmental parameters had a stronger effect on endangered species abundance rather than on their richness. The *mesohabitat model* (Table 4) showed that the sampling month and the sampling method significantly affected the abundance of endangered species, with higher values in April, June and July than in August and September ($p < 0.0055$), and higher values in T than in MH samples ($p = 0.0325$). In April (Fig. 3), the highest abundances were found in MH samples (in both UPs and DWs). Peaks in the abundance of endangered species were observed in the Arrestra stream (UP and DW sites) in two lateral pools connected with the main flow and shaded by the riparian vegetation. During SUMMER (Fig. 4), in the UP sites the highest median values were reached in the MH samples, except in June, in which the median was slightly higher in T samples. In the DW sites, T generally hosted the highest abundance of endangered species. However, if we consider the extreme values, we can observe that peaks of endangered taxa were mainly observed in the MH samples of UP sites, but also in DW sites.

Considering the *microhabitat model* (Table 4), significant differences were observed between the two flow velocity categories, with higher values in running waters (group 1) than in standing waters (group 0) ($p = 0.0360$). A negative significant effect of macrophyte coverage was also detected, suggesting that microhabitats with standing waters, normally hosting a high percentage of macrophytes, are less suitable for sheltering endangered species.

DISCUSSION

Mediterranean freshwater ecosystems are currently facing a huge species loss, thus calling for evaluation of their biodiversity status (Dudgeon et al. 2006). In this context, diatoms represent a poorly investigated group of freshwater organisms (Novais et al. 2014). In our study, we applied an integrated sampling approach, which allowed us to investigate microhabitats that are usually underrepresented. We highlighted environmental parameters that favor the abundance of endangered taxa and we better defined the ecological preferences of certain threatened species.

Firstly, the CA results showed that diatom communities were highly separated at the stream level in terms of community composition, especially during the low flow season. This result is in accordance with Tornés & Ruhí (2013), who observed a higher frequency of idiosyncratic species in hydrologically disturbed rivers, and with Novais et al. (2014), who observed that diatom species in permanent watercourses are also present in temporary watercourses but not vice versa. This highlighted the peculiarity of Mediterranean rivers and underlined the need to redefine the Red List at the hydroecoregional level. Indeed, even if some species are commonly found in other hydroecoregions, they may become rare in the Mediterranean area since they could be relegated to a single watercourse. According to our results, drought in Mediterranean rivers seems to be the main motive for the reduction of the abundance of endangered species. Indeed, as demonstrated by our *mesohabitat model*, we observed a reduction in the abundance of endangered taxa in August and September, and the standard samples, performed in the main stream channel, always hosted the highest abundance of Red List species. The results of the *microhabitat model* also confirmed this trend, as the presence of flowing water (> 0.20 m/s) proved to be a determinant parameter for guaranteeing a high abundance of endangered species.

In Mediterranean streams, terrestrial species represent key organisms in the recolonization of watercourses following drought. As mentioned previously, Souffreau et al. (2013) observed that only species that tolerate low values of moisture were able to survive desiccation through resting cell formation, sometimes as vegetative forms. In Mediterranean rivers, this strategy would greatly help diatoms to face harsh conditions during the summer months, increasing the survival rate and favoring the recolonization after the return of waters. For this reason, particular importance should be given to the presence of these taxa in rivers characterized by hydrological disturbance, and their inclusion in the Red List as threatened taxa should be considered for temporary rivers. In this research, we recorded only one strictly terrestrial diatom, namely *Adlafia bryophila*

When the ecological requirements of endangered species are considered, we can highlight the importance of pool microhabitats, which are normally excluded from standard sampling protocols. *A. lineare* seems to be highly widespread in the temporary streams of the Mediterranean hydroecoregions (Novais et al. 2014), and from our results we confirmed its preference for oligotrophic rivers, in circumneutral to alkaline waters and low-moderate conductivity values (Van de Vijver et al. 2011). *Achnanthidium gracillimum* is considered a sensitive species and is generally found in calcareous rivers with low organic matter and nutrient content (Ponander and Potapova 2007; Hofmann et al. 2011; Bey and Ector 2013). In general, both these species were more abundant in shallow pools during both the intermediate and the low flow season. Considering *Nitzschia gessneri*, little information is available on its ecology. We observed that it preferred pool microhabitats and calcareous substrates, without showing high relative abundance, as also observed by Hofmann et al. (2011). Similar preferences were also noted for species belonging to other threatened categories (e.g. *Gomphonema tergestinum, G. lateripunctatum* and *Navicula novaesiberica*). We can therefore suggest that during the intermediate flow, as well as in those sites characterized by permanent flow all over the year, lentic microhabitats represent suitable and favorable niches in which endangered taxa can be hosted. Therefore, the sampling approach based on microhabitats enhanced the possibility to collect rare and endangered species compared to standard methods, thus contributing to a greater opportunity for increasing the knowledge on their distribution and ecological requirements. Conversely, during the hydrological disturbance, the parts of the river connected with the main flow, where the standard sampling was performed, sheltered the highest number of endangered individuals, while the presence of isolated pools and/or characterized by intermittent flow, negatively affected the presence of threatened taxa. Despite this, the presence of exceptions, represented here by extreme values in the number of endangered individuals during summer, demonstrated the importance of the preservation of aquatic habitats during the dry season.

During this study, two species with invasive behavior were recorded, namely *D. geminata* and *A. delmontii*, which both increased in abundance during the summer season. The inclusion of *D. geminata* among the "threatened with extinction" taxa is surprising. This classification is probably derived from the original description of *D. geminata* that considered its diffusion as being limited to mountainous pristine and oligotrophic habitats of the circumboreal regions (Blanco and Ector 2009). However, the recent spread of this species all over the world and in different kinds of freshwater habitats (Blanco and Ector 2009; Falasco and Bona 2013), together with the nuisance effect of its blooms, has led us to state that a reconsideration of its conservation status is needed. Concerning *A. delmontii*, this species appeared in France for the first time in 2007, when it was recorded with low percentage relative abundance, and in 2012 it reached more than 60% peaks of abundance (Pérès et al. 2012). To date, the only published records on *A. delmontii* are with respect to its distribution in France. In our study, *A. delmontii* was exclusively collected in the Argentina stream and showed a significant increase of relative abundance from April, when it was absent, to September, when it dominated the communities in some cases reaching almost 70% of relative abundance.

In our study, emerging metrics, such as ecological guilds appear not to be reliable response variables for evaluation of this kind of hydrological disturbance, as flow probably plays a secondary role in shaping their relative proportions. Indeed, nutrient content mainly drives diatom functional traits (Larson and Passy 2012; Novais et al. 2014). On the other hand, the percentage of endangered species emerged as a promising and important metric towards quantification of the hydrological disturbance caused by natural and anthropic pressures. Unfortunately, unclear or missing classifications of conservation status for several species still persist, and our work has shown that there is a need to update the Red List.

CONCLUSIONS

Diatom communities in Mediterranean rivers shelter a good proportion of species that are considered as threatened at different levels. However, a high percentage of the recorded species is still not classified according to the Red List, highlighting once again the need for its update and extension. Endangered species responded to hydrological disturbance more than functional traits, with the tendency to decrease their abundance with increasing harsh conditions. Sites characterized by permanent water flow throughout the year hosted the highest percentage of endangered species, especially in stretches where heterogeneity is preserved. Thus, the unconventional sampling approach adopted during our research, which involved highly differentiated microhabitats, permitted the recording of a higher number of rare and threatened taxa, which would have been absent if only traditional procedures were followed.

Future research on this study area should possibly consider pluriannual samplings in order to account for interannual variability and future trends. In light of our results, diatoms can provide useful information to improve river management practices when faced with an increasing water scarcity scenario. Primarily, the heterogeneity of the river habitat should be preserved and enhanced. This must be carried out in conjunction with the maintenance of flowing waters (with a minimum velocity of 0.20 m/s), which is a key factor for increasing the abundance of threatened taxa. In drought conditions, the maintenance of isolated pools surrounded by dry riverbeds is still very important, as they have to be considered as unique *refugia* for benthic diatom communities.

Compliance with Ethical Standards

Conflict of interest: the authors declare that they have no conflicts of interest

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Figure captions

Fig. 1 Streams and sites locations. Squares represent upstream sites; circles represent downstream sites

Fig. 2 CA representation of diatom communities collected during SPRING (a.) and SUMMER (b.)

Fig. 3 SPRING: relative abundance of endangered species (sum of the categories "threatened with extinction", endangered", "probably endangered", "rare" and "decreasing") in the up- (UP) and downstream (DW) sites; further distinction between transect (T) and microhabitat (MH) approaches is provided

Fig. 4 SUMMER: relative abundance of endangered species (sum of the categories "threatened with extinction", endangered", "probably endangered", "rare" and "decreasing") in the up- (UP) and downstream (DW) sites; further distinction between transect (T) and microhabitat (MH) approaches is provided

Tab. 1 Physical-chemical parameters and diatom biological attributes detected during intermediate (SPRING) and low (SUMMER) flow, in both up- and downstream (UP and DW) sites. Mean values and standard deviations are displayed in the table

Tab. 2 Percentages of species belonging to different conservation status. Red List columns refer to conservation status defined in Lange-Bertalot & Steindorf (1996): 1 = threatened with extinction. 2 = severely endangered. 3 = endangered. G = probably endangered. R = rare. V = decreasing. * = at present not considered threatened. ? = not threatened. • = common. Z = not listed. D = data scarce. n° samples = Total number of samples. n° species = Total number of identified species

Tab 3 Results of the Indicator Species Analysis (ISA) on the following groups: sampling MONTH (April, June, July, August, September); RIVER (Argentina, Impero, Merula, Quiliano, Vallecrosia); SITE LOCATION (UP = upstream, DW = downstream); SAMPLING METHOD (T = transect, MH = microhabitat); FLOW VELOCITY (velocity ≤ 0.20 m/s, velocity > 0.20 m/s); WATER DEPTH (depth ≤ 0.25 m, depth > 0.25 m); SHADE (present, absent); ISOLATION (connected to the main channel, isolated habitat); DOMINANT SUBSTRATE (boulders > 40cm diameter, cobbles 40-6 cm diameter, pebble and sand <6 cm diameter); MACROPHYTES (coverage ≤ 50%, coverage > 50%); ALGAE (coverage ≤ 50%, coverage > 50%). Complete list of the diatom codes is shown in the ESM_2. Significant p values for each species are reported in parenthesis.

Tab 4 Results of the effect of fixed factors in the *mesohabitat* and *microhabitat models* as inferred with GLMMs; results for both the number of Red List taxa (N° of RL taxa) and the abundance of individuals (Abundance of RL taxa) are reported (F = F-value; P = P-value; Est = estimates)

Tab. 1

		SPRING		SUMMER	
		UP	**DW**	**UP**	**DW**
		mean ± sd	**mean ± sd**	**mean ± sd**	**mean ± sd**
physical-chemical parameters					
	DO (%)	100.6 ± 1.25	105.6 ± 14.14	99.1 ± 11.37	99.9 ± 34.10
	pH	8.96 ± 0.2	8.95 ± 0.27	8.39 ± 0.53	8.22 ± 0.50
	TEMP (°C)	13.3 ± 2.13	15.5 ± 3.82	20.1 ± 2.03	21.1 ± 2.02
	TSS (mg/l)	1.78 ± 1.27	5.85 ± 2.61	2.61 ± 3.65	3.41 ± 35.09
	COND (µS/cm)	327 ± 151	352 ± 139	400 ± 160	406 ± 182
	N-NO_3 (µg/l)	750 ± 309	960 ± 314	380 ± 210	400 ± 913
	SRP (µg/l)	29 ± 7	15 ± 13	10 ± 13	7 ± 29
biological attributes					
	n° species	22.8 ± 5.23	22.3 ± 6.68	27.6 ± 6.69	24.9 ± 6.72
	Shannon Index	2.64 ± 0.59	2.53 ± 0.70	3.05 ± 0.56	2.85 ± 0.75
ecological guilds	**low profile (%)**	67.25 ± 18.57	64.13 ± 20.56	77.70 ± 11.25	70.09 ± 19.79
ecological guilds	**high profile (%)**	15.00 ± 11.15	13.72 ± 9.98	8.15 ± 6.21	15.73 ± 11.44
ecological guilds	**motile (%)**	17.55 ± 18.27	21.54 ± 19.26	13.64 ± 8.64	12.65 ± 14.35
ecological guilds	**planktic (%)**	0.20 ± 0.43	0.61 ± 1.45	0.51 ± 1.06	1.53 ± 6.18
	colonial (%)	11.93 ± 10.30	11.98 ± 8.67	6.23 ± 6.11	11.50 ± 10.84
	adnate (%)	5.67 ± 9.19	4.46 ± 6.00	17.83 ± 22.16	8.37 ± 12.86
	peduncolate (%)	70.09 ± 21.52	70.22 ± 21.07	66.60 ± 25.16	76.45 ± 20.71

Tab. 2

RED LIST		number of species (relative % of abundance)			
CODE	**STATUS**	**SPRING**	**TOTAL**	**SUMMER**	**TOTAL**
1	**threatened with extinction**	0.60		0.41	
2	**severely endangered**			0.41	
3	**endangered**	3.57		4.56	
G	**probably endangered**	2.98		2.49	
R	**rare**	0.60		0.41	
V	**decreasing**	2.98	**10.71**	5.81	**14.11**
*****	**at present not considered threatened**	23.21		19.50	
?	**not threatened**	33.33		29.88	
•	**common**	1.19	**57.74**	1.24	**50.62**
z	**not listed**	29.17		32.37	
D	**data scarce**	2.38	**31.55**	2.90	**35.27**
	n° samples	***126***		***240***	
	n° species	***171***		***241***	

Tab. 3

	GROUP DESCRIPTION	**taxa (p value)**
MONTH	*April*	ENVE (0.001); GCBC (0.001); GTER (0.001); GOLI (0.001); NLIN (0.001); NGRE (0.001); ADMS (0.001); GMIC (0.001); DEHR (0.002); CAFF (0.001); FCCT (0.001); FSAP (0.003); MCIR (0.003); FPEL (0.001); FARC (0.014); HPDA (0.006); SBKU (0.006); GANT (0.014); NACI (0.022)
	June	GVID (0.021); FDEL (0.043); SANC (0.036)
	July	CNCI (0.007); CALO (0.008); FLAT (0.037)
	August	GRHO (0.001); NIZT (0.024)
	September	DCOF (0.001); SSVE (0.001); CTUM (0.001); SBND (0.024); CNLP (0.010)
RIVER	*Argentina*	ADPT (0.001); ADMO (0.001); DEHR (0.001); NILA (0.001); NTAB (0.001); DGEM (0.001); FVAU (0.001); NSBN (0.001); ADTH (0.001); DEHT (0.001); ADCT (0.001), ADLA (0.003); FARC (0.010); ESAB (0.044)
	Impero	ADEU (0.001); FRCP (0.001); RABB (0.001); GITA (0.001); DCOF (0.001); CAFF (0.001); RUNI (0.001); SBND (0.001); EOMT (0.001); SSVE (0.013); NMIC (0.049); CNLP (0.012); APAB (0.015); FCCT (0.043); EPRO (0.035)
	Merula	DKUE (0.001); SACU (0.001); CSUT (0.001); GLAT (0.001); NGES (0.001); CEXF (0.001); CDTG (0.001); GCBC (0.001); GVID (0.001); FALP (0.001); NRAD (0.003); EUFL (0.002); CLAE (0.00); GANT (0.018); FDEL (0.040);
	Quiliano	CLNT (0.001); RSIN (0.001); PTLA (0.001); FPRU (0.001); PLFR (0.001); COPL (0.001); NINC (0.001); NIAR (0.001); NCRY (0.001); MPMI (0.001); FRUT (0.001); CTRO (0.001); CPTG (0.001); ADGL (0.001); ADSU (0.001); MVAR 0.001); EULA (0.001); NSPD (0.001); SSEM (0.001); NYCO (0.001); FSAP (0.001); CMEN (0.001); ADMS (0.002); GACU 0.001); CTUM (0.001); CPLA (0.001); GACC (0.001); CNCI (0.005); CALO (0.010); HPDA (0.009); SBKU (0.009); ADTG 0.034); GDEC (0.033); KCLE (0.043)
	Vallecrosia	NVEN (0.001); NCTO (0.001); GPUM (0.001); FMES (0.001); AOVA (0.002); CVUL (0.001); SANC (0.041)
SITE LOCATION	*UP*	DTEN (0.001); NCTO (0.001); PSBR (0.001); ADAM (0.001); SEBA (0.001); GITA (0.014); EULA (0.015); FMES (0.014); SSEM (0.023); GACU (0.002); NRAD (0.027); NSBN (0.049); APAB (0.021); AOVA (0.021); GANT (0.041); CVUL (0.043); DEHT (0.042)
	DW	NINC (0.015); GLAT (0.006); UBIC (0.001); FPRU (0.001); GPUM (0.001); ADGL (0.001); FRUT (0.009); CAFF (0.024); NPAL (0.005); CTRO (0.014); NYCO (0.026); FCAT (0.009); CNCI (0.028)
SAMPLING METHOD	*T*	FVAU (0.016); FGRA (0.022); FSAP (0.042); ADLA (0.024); CETG (0.019); NIZT (0.032); SVTL (0.034)
	MH	-
FLOW VELOCITY	*$V \leq 0.20$ m/s*	GCAP (0.011)
	$V > 0.20$ m/s	DMON (0.001)
WATER DEPTH	*depth ≤ 0.25 m*	ADGL (0.032)
	depth > 0.25 m	DGEM (0.049); NSBN (0.007); GVID (0.023); ENLB (0.047); ECMT (0.037)

	present	FRCP (0.015)
SHADE	*absent*	ACLI (0.022); CLNT (0.017); DEHR (0.005); NCTO (0.033); CPTG (0.017); COPL (0.012); ADAM (0.004); GACU (0.001); EULA (0.007); NRAD (0.007); FMES (0.023); SSEM (0.035); FARC (0.010); AOVA (0.037); DMES (0.035); GDEC (0.031); CALO (0.042)
ISOLATION	*connected*	CLNT (0.045); NGRE (0.032); PTLA (0.049)
	isolated	DKUE (0.001); ESUM (0.001); GLAT (0.001); SACU (0.001); FPEM (0.002); CSUT (0.002); UBIC (0.003); SSTM (0.027); CDTG (0.002); ECES (0.001); ADMO (0.010); GOMP (0.003); GPUM (0.027); NSBN (0.010); EUFL (0.012); FDEL (0.001); CLAE (0.010); DPAR (0.026); CBAM (0.017); EUNO (0.048)
DOMINANT SUBSTRATE	*boulders (> 40cm diameter)*	NILA (0.001); CPAR (0.001); SSTM (0.001); DEHR (0.001); NCTO (0.001); DTEN (0.001); ECAE (0.001); FMES (0.001); DVUL (0.002); NVEN (0.001); DGEM (0.001); SEBA (0.001); PSBR (0.003); AOVA (0.001); DEHT (0.001); FGRA (0.003); NSBN (0.006); ADCT (0.001); ADJK (0.013); FARC (0.003); FAUT (0.023); ECMT (0.005); CVUL (0.013); NATG (0.021); ESAB (0.026)
	cobbles (40-6 cm diameter)	ADMO (0.002); RABB (0.023); GPUM (0.004); DCOF (0.024)
	pebble and sand (<6 cm diameter)	CLNT (0.001); RSIN (0.001); PTLA (0.001); NCRY (0.001); UBIC (0.001); NGRE (0.002); COPL (0.001); PLFR (0.006); CTRO (0.001); MPMI (0.001); GLAT (0.043); ADSU (0.001); MVAR (0.038); FPRU (0.013); ADGL (0.006); EULA (0.003); FRUT (0.008); ADMS (0.001); FSAP (0.022); CTUM (0.006); CMEN (0.028); GACU (0.004); NSPD (0.011); NREC (0.029); HPDA (0.042); SBKU (0.038)
MACROPHYTES	*coverage ≤ 50%*	RSIN (0.002); CLNT (0.001); CPTG (0.001); COPL (0.001); FPRU (0.002); FSAP (0.001); DCOF (0.005); MPMI (0.034); GOMP (0.001); NMIC (0.038); CPLA (0.024); ENLB (0.038); HPDA (0.050); SBKU (0.050)
	coverage> 50%	DMON (0.002); DEHR (0.048); GCAP (0.007); FMES (0.002); DGEM (0.023); FGRA (0.010); FAUT (0.042); FCAT (0.011)
ALGAE	*coverage ≤ 50%*	ACLI (0.005); RSIN (0.001); CLNT (0.001); NIAR (0.003); CPTG (0.001); COPL (0.001); FPRU (0.002); MPMI (0.011); FSAP (0.002); EULA (0.004); DCOF (0.020); GOMP (0.003); GACU (0.008); CPLA (0.036); EUFL (0.048)
	coverage> 50%	DMON (0.001); DEHR (0.018); GCAP (0.004); FMES (0.001); FGRA (0.003); FAUT (0.010); FCAT (0.006); AOVA 0.022); ADCT (0.026); ADLA (0.027)

Tab. 4

MESOHABITAT MODEL					MICROHABITAT MODEL						
Variable	**N° of RL taxa**		**Abundance of RL taxa**		**Variable**	**N° of RL taxa**			**Abundance of RL taxa**		
Month	$F_{4,268}$ = 1.47	P = 0.2108	$F_{4,268}$ = 9.62	P < 0.0001	*Macrophytes*	Est = -0.0014	$F_{1,272}$ = 1.15	P = 0.2839	Est = -0.0061	$F_{1,272}$ = 5.79	P = 0.0163
Sampling site	$F_{1,268}$ = 0.72	P = 0.3970	$F_{1,268}$ = 0.29	P = 0.5926	*Flow velocity*	Est = 0.1187	$F_{1,272}$ = 1.55	P = 0.2139	Est = 0.4190	$F_{1,272}$ = 4.44	P = 0.0360
Sampling method	$F_{1,268}$ = 0.62	P = 0.4307	$F_{1,268}$ = 4.62	P = 0.0325	*Water depth*	Est = 0.0020	$F_{1,272}$ = 0.53	P = 0.4659	Est = -0.0076	$F_{1,272}$ = 1.08	P = 0.1719
Disturbance	$F_{1,268}$ = 0.00	P = 0.9494	$F_{1,268}$ = 0.09	P = 0.7605							