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Abstract

The main objective of the present study was to explore the potential link between acetylcholinesterase (AChE) activity and burrowing behaviour of the ragworm *Hediste diversicolor*, which may have consequences at higher levels of biological organisation. Two complementary studies were conducted. AChE activity, at the sub-individual level, and behavioural responses, at the individual level, were evaluated in worms from the Loire estuary (France), whereas density and biomass of *H. diversicolor* were determined at the population level. A Spearman positive correlation between both biomarkers (AChE and burrowing) suggested that inhibition of AChE activity was linked to behaviour impairments. At the population level, lower AChE and behaviour activities were detected in worms corresponding to lower population density and biomass. These results provide direct empirical field evidence demonstrating the sensitivity of behaviour of *H. diversicolor* as a biomonitor of estuarine health status assessment.

Key words: *Hediste diversicolor*; acetylcholinesterase; population density; behaviour; Adverse Outcome Pathway.

Estuaries, as transitional zones, provide a wide range of vital nesting and feeding habitats and ecosystem services (Dallas and Jha, 2015), but since many decades, they are affected by multiple anthropogenic activities, leading to the release of various types of contaminants. To estimate the health status of ecosystems, biomonitoring programs aimed to classically combine chemical and biological approaches (e.g. multibiomarker approach) and ecological indicators. Conventional monitoring techniques are mainly based on the determination of the contaminants' concentrations in sediments and water. Evaluation of chemical contamination is a complex task, as estuaries often present a mixture of contaminants and their metabolites, some of which are costly or not yet accessible to analyze. It is widely recognised that chemical data alone can only provide partial information; for example, the unpredictability of the processes affecting the speciation of pollutants may alter their bioavailability and therefore their toxicity (Birch, 2018). For this reason, the integration of biomarker responses with chemical and ecological approaches on sentinel species allows evaluation of the toxicity of mixtures of pollutants and sub-lethal effects even at low concentrations (Amiard-Triquet et al., 2015). Biomarkers have been widely used to assess contaminant exposure and its effects in marine species such as the ragworm *Hediste diversicolor*, which plays an important role in the structure and functioning of marine ecosystems. However, biomarkers are subjected to the influence biotic and abiotic confounding factors (e.g. temperature, salinity, size/weight and reproductive status). Thus, to use these biomarkers in biomonitoring programs, it is necessary to previously determine baseline levels in species of interest, which is currently

challenging. Recently, Barrick et al. (2016; 2018) determined baseline assessment criteria (BAC) and environmental assessment criteria (EAC) of biochemical biomarkers (energy reserves, catalase: CAT, glutathione S transferases: GST, acetylcholinesterase: AChE and thiobarbituric acid reactive substances: TBARs) in *H. diversicolor* from a reference site (Durou et al., 2007). Regarding burrowing behaviour, Kalman et al. (2010) showed no influence of confounding factors (weight, salinity and grain size) on burrowing behaviour in *H. diversicolor*.

Field studies aiming at establishing a link between biomarkers at a low level of organisation and possible effects at a higher level of organisation (e.g. population and community) remain to be conducted. The adverse outcome pathway (AOP) approach proposed by Ankley et al. (2010) aims to break through the gap linking direct molecular initiating events with adverse outcomes at a higher level of organisation. AChE, a core biomarker of neurotoxicity, is well recognised as a biomarker of ecological relevance, as its activity inhibition observed in laboratory or field studies may lead to physiological and behavioural impairments in organisms exposed to not only pesticides but also metals, polycyclic aromatic hydrocarbons (PAHs), pharmaceutical products or algal toxins (Lionetto et al., 2013; Sturve et al., 2016; Lehtonen et al., 2003; Kankaanpää et al., 2007; Zhang et al., 2016).

Currently, the most robust of the developing pathway is the AOP linking AChE inhibition to acute mortality (Russom et al., 2014). This AOP has been proposed to support integrated approaches for testing and assessment (https://aopwiki.org/aops/16). However, there are many biochemical, behavioural, and physiological processes associated with the link between AChE inhibition and acute mortality of organisms and, consequently, possible density depletion of the species considered. The Loire estuary is a multi-contaminated estuary displaying a diffusive

74 pollution due to heavy metals, pesticides, PAHs and PCBs (Blanchet-Letrouvé et al.,
75 2013), and AChE inhibition in organisms has been often linked to the presence of these
76 contaminants. The main objective of the present study was to explore in the ragworm
77 *H. diversicolor* from the Loire estuary (France), the potential link between AChE activity
78 and burrowing behaviour that may have consequences at higher levels of biological
79 organisation.
80 The sampling site was located on the largest mudflat of the polyhaline domain of the
81 Loire estuary ('Les Brillantes') on the south shore downstream Paimboeuf (Fig. 1). Two
82 zones were selected: the first one (Site 1: 47°16'55.70"N; 2° 3'47.60"W) was located
83 in the upper intertidal area of the mudflat, 20 m from the shore, located close to an
84 active eroded cliff (Thibault de Chanvalon et al., 2016). The second one, (Site 2:
85 47°17'3.77"N; 2° 3'42.87"W) was located in the middle intertidal area. Two sampling
86 campaigns were conducted in May 2013 (after the flood period) and February 2014
87 (during the flood period), respectively.

88 For AChE activity determinations, 10 worms (*H. diversicolor*) in the same range of wet
89 weight (mean S1: 0.220±0.114 g; mean S2: 0.187±0.121 g) were gently collected by
90 hand, kept in wet sediment from their site of origin, immediately frozen at the laboratory
91 and then kept at -80°C until analysis. AChE activity was determined using the method
92 described in Buffet et al. (2011). Briefly, whole worm tissues were individually
93 homogenised in 80 mM Tris-HCl pH 7.2, 200 mM NaCl, 1 mM Dithiothreitol
94 supplemented with protease inhibitor cocktail according to the manufacturer's
95 instructions. The homogenates were centrifuged at 9000 *g* for 30 min at 4°C, and
96 supernatants were collected for protein (Bradford, 1976) and AChE activity
97 determinations. Ten microlitres of the supernatant was added in triplicate to 190 µl 10
98 mM DTNB and 100 µL 100 mM acetylthiocholine into a 96-well microplate. The

99 reactions were read at 412 nm for 3 minutes. Results were expressed as nmoles of
100 thiocholine produced per min and per mg protein (nmoles min^{-1} mg^{-1} protein).
101 Concerning behavioural experiments, burrowing tests were set up in a dark
102 temperature-controlled room (15°C at the time of collection). Worms (*H. diversicolor*)
103 were submitted to burrowing tests as previously described by Bonnard et al. (2009).
104 Sediments from each site were homogenised by hand one day before experimentation.
105 Briefly, burrowing behaviour was studied by placing individually 20 worms on
106 sediments from their site of origin and recording their positions every 2 min for 30 min.
107 Automated behavioural tests were carried out with the Multifreshwater Biomonitor®
108 (MFB) from LimCo International according to the methodology previously described
109 and validated by Buffet et al. (2013). *H. diversicolor* movements inducing signals within
110 the range of 0.5 Hz were identified as undulation, whereas signals induced between
111 1.0 and 2.0 Hz indicated head movements and feeding activity. One individual per
112 chamber (n=7) was placed on sediment surface and allowed to acclimate 1 h before
113 MFB recording during 7 h. One control chamber without any organisms was used for
114 measuring background noise signals. Concerning density measurements, four
115 sediment samples from both areas (Site 1 and Site 2) were randomly collected at low
116 tide using plastic cores (10 cm x 35 cm) at a distance of 10 m from each other.
117 Sediment was sieved through a 1 mm mesh to collect and count worms. Fresh weight
118 of *H. diversicolor* individuals was determined to establish biomass. An average of 4
119 biomass replicates was calculated as the sum of fresh weight (g FW) divided by the
120 sediment surface sampled (m^2) and expressed as FWg m^{-2}. Statistical analysis for
121 burrowing kinetics was performed on R (R Core Team 2014) using a generalised non-
122 linear model with α as burrowing rate (h^{-1}). This generalised non-linear model with a
123 conditional binomial stochastic part was fitted to each data set by using Bayesian

124 inference as described in Buffet et al. (2014). For all the other parameters, results are
125 presented as mean ± standard deviation, and statistical analyses were performed
126 using XLSTAT 2012. Normality was assessed using the Shapiro–Wilk test. To define
127 significant differences between sites for each sampling date, normally distributed data
128 were analyzed by one-way ANOVA (multiple comparisons of mean values) followed
129 by Tukey's HSD post hoc method. When normality was not established, the non-
130 parametric Kruskal–Wallis and Mann–Whitney comparison tests were used. The level
131 of significance was established at $p < 0.05$. A Spearman's correlation (Daniel, 1990)
132 was computed to quantify the relationship between AChE activity and behavioural
133 toxicity parameter values. The Spearman's rank-order correlation is a non-parametric
134 correlation coefficient ρ, measuring strength and direction of the monotonic
135 relationship between AChE activity and MFB. The statistical significance test of the
136 Spearman correlation was performed to investigate whether the null hypothesis 'H0:
137 There is no monotonic association between AChE and the behavioural toxicity
138 parameter MFB' can be rejected.

139 Lower AChE activity was found in worms *(H. diversicolor*) sampled from Site 2
140 (10.07±5.80 nmole.min^{-1}.mg^{-1} proteins) than those in Site 1 (23.34±8.90 nmole.min^{-}
141 ^{1}.mg^{-1} proteins). AChE activities measured in *H. diversicolor* from Site 2 (compared to
142 Site 1) showed 40% and 79.5% of inhibition in May 2013 and February 2014,
143 respectively (Table 1). Results showing the positions of AChE activity measurements
144 in worms from both areas (Site 1 and Site 2) according to the four colour traffic light
145 previously identified by Barrick et al. (2018) (red: lethal effects expected; orange:
146 sublethal effects expected; yellow: range expected for non-impacted site; green:
147 evidence of good health) are illustrated in Figure 2. In February, AChE values were in
148 the range where sub-lethal effects (worms from Site 1) and lethal effects (worms from

149 Site 2) were expected. In May, AChE values for worms from Site 1 were in the yellow
150 range expected for non-impacted site, whereas those for worms from Site 2 were in
151 the range where sub-lethal effects were expected. Results of burrowing kinetics carried
152 out with *H. diversicolor* from both areas (Sites 1 and 2), allowed to burrow in sediment
153 from their site of origin, are shown in Table 2. Burrowing kinetics of worms from Site 2
154 were significantly impaired in February 2014 compared to those in Site 1. Behavioural
155 differences between worms from both sites measuring with the MFB are illustrated in
156 Table 2. Significant frequency decrease ($p=0.021$) corresponding to body undulation
157 (0.5 Hz) was observed for worms from Site 2 compared to those from Site 1. Decreases
158 of head movements and feeding activity (between 1.0 and 2.0 Hz) were also observed
159 for worms from Site 2 *vs* those from Site 1 ($p=0.066$). The mean Spearman's
160 correlation coefficient of 0.5404 revealed a positive correlation ($p=0.0686$) between
161 AChE activities and MFB values. Data of densities and biomass of *H. diversicolor* at
162 both sites are shown in Table 3. Densities and biomass of ragworms collected at Site
163 1 were significantly higher than those at Site 2 for both sampling dates (May 2013 and
164 February 2014).

165 The AOP approach initially proposed by Ankley et al. (2010) aims to link direct
166 molecular initiating events with adverse outcomes at a higher biological level of
167 organisation relevant to risk assessment. The usefulness of this approach for
168 biomarker-based environmental risk assessments has been recently demonstrated
169 using aquatic organisms (Wuk Lee et al., 2015). Effects observed at the individual and
170 subcellular level (defence and damage responses: oxidative stress, immunotoxicity,
171 neurotoxicity, genotoxicity and apoptosis) can be propagated upwards through
172 increasing levels of organisation. Behavioural damages could find their origin in
173 different biochemical and physiological impairments such as sensory system

disruption, endocrine disruption, neurotoxicity or energy disturbances (Amiard-Triquet and Amiard, 2012) at a lower level and population effects at a higher level of organisation. In the laboratory, numerous studies showed that decreased in AChE activity induced behavioural disturbances in different species exposed to several contaminants (Sismeiro-Vivas et al., 2007; Ren et al., 2015; Haverroth et al., 2015; Geraudie et al., 2016). However, in the field, it is necessary to conduct studies able to demonstrate a causal relationship between behavioural responses and effects at higher levels of biological organisation such as population effects (Amiard-Triquet et al., 2015). In the present study, we investigated, the potential links between three different levels of biological organisation studied, precisely: sub-individual (acetylcholinesterase activity), individual (burrowing behaviour) and population (density and biomass) of worms. Results showed, at the biochemical level, a significant lower value of AChE activity in worms (*H. diversicolor)* from Site 2 (decrease of 40% in May 2013 and of 79% in February 2014 *vs* Site 1). Previously published studies reported that a reduction of 20% in AChE activity in fish and invertebrates indicates exposure to neurotoxic compounds (organophosphorus, pesticides), whereas depletion of AChE activity by 20-50% indicates sublethal impact (Amiard-Triquet et al., 2015). Mudflat sediments act as the final sink for various contaminants depending on their properties. Sorption of organic contaminants on sediments is controlled by grain size and organic matter content (Arslan-Alaton and Olmez-Hanci 2013). It has been demonstrated that bioavailable metals and organic contaminant contents were low in organic carbon enriched sites, tending to be adsorbed on fine-grained sediments (Pelletier et al., 2011; Morrison et al., 2011). Site 1, compared to site 2, was composed of slightly fine sediment and was slightly enriched in total organic carbon known to have strong sorption capacities of contaminants (Luthy, 2004; Thibault de Chanvalon

et al., 2016). Moreover, Site 1 was located in an active eroded cliff where vegetation and rhizosphere could develop synergies with microorganisms increasing pollutant removal rates such as PAHs (Thibault de Chanvalon et al., 2016). At the individual level, burrowing kinetics of worms from Site 2 were significantly impaired compared to that in worms from Site 1. Schriks et al. (2006) used the MFB in *Xenopus laevis* as a sensitive and useful tool to quantify behavioural effects induced by toxic compound. In *H. diversicolor*, the use of MFB allows a more sensitive estimation of behavioural activities of worms, as differentiation between body undulation, head movements and feeding activity of worms can be assessed (Buffet et al., 2013). In this work, results showed lower body undulation of worms from Site 2 (*vs* Site 1). Statistical analysis (Spearman correlation) performed on the set results of AChE activities and behaviour (MFB) showing a positive correlation between both biomarkers (biochemical and behavioural) suggested that lower AChE activities were associated with lower behaviour of worms (and inversely). Feeding behaviour disturbances have been previously shown in *H. diversicolor* from the multipolluted Loire estuary as compared to controls from a reference site (Fossi-Tankoua et al., 2012). Recently, Harayashiki et al. (2016) demonstrated behavioural and AChE biochemical alterations in *Penaeus monodon* post larvae diet-exposed to inorganic mercury, reducing swimming activity. In the invertebrate *Gammarus fossarum,* Xuereb et al. (2009) showed that AChE inhibition levels greater than 50% led to population depletion. At the population level, worms exhibiting lower AChE activities and behaviour corresponded to those collected from the site (Site 2) where lower population densities were found. Indeed, densities of *H. diversicolor* showed a significantly contrasting status between both sites with significantly higher densities (280.61±325.69 to 634.57±294.45 ind.m^{-2}) in the upper intertidal Site 1 and lower densities in the middle intertidal Site 2 (25.51±23.29 to

66.96±28.28 ind.m^{-2}). These observations cannot be relied on a patchy distribution of worms on the mudflat, as the distribution of *H. diversicolor* is known to be rather relatively uniform regarding the regularly distributed openings of burrows on the sediment surface (Kristensen et al., 2013). However, it has been demonstrated in *H. diversicolor* a double-way relationship between reworking behaviour and density as a decrease in individual sediment reworking activity when density increase (Duport et al. 2006) and at high levels of algal enrichment (Murray et al. 2017). Moreover, at high abundances, it has been shown that territorialism by this ragworm species is a mechanism underplaying trophic competition, while both phenomena seem to disappear at low population density (Kristensen et al. 2013). Site 1 was also enriched in living benthic foraminifera with a total density of 2199.35 individuals per 100 cm^{-3} compared to that at site 2, where a density of only 48 individuals per 100 cm^{-3} was found (Mojtahid et al., 2016). These authors correlated the microphytobenthic biofilm development on 'Les Brillantes' mudflat with the foraminifera species abundance, as it constitutes important quantities of food resources. Cardoso et al. (2004) showed that *H. diversicolor* had greater abundance in areas where algae were available, as a food source and a refuge from predators. Christensen et al. (2000) showed that an abundant supply of nutritious food (phytoplankton) increased *H. diversicolor* metabolism considerably. To cope, chemical stress is metabolically costly leading to a shift in allocation of energy with implications for growth and reproduction processes (Calow and Forbes, 1998). Thus, in the present study, it could be suggested that lower food resources and energetic cost of defence mechanisms to cope with chemical stress in worms from Site 2 *vs* Site 1 could lead to a reduction of the most energetic expensive individual processes, growth and reproductive potential, resulting in detrimental effects observed at the population level.

In conclusion, the contrasted status of *H. diversicolor* densities between both sites could be partly explained by AChE activity decrease and behavioural impairments of worms from the middle intertidal mudflat (Site 2) compared with the upper intertidal site (Site 1), which could result in the detrimental population effects observed. This study brings direct empirical field evidence supporting impact of AChE inhibition and behaviour at the population level demonstrating the sensitivity of behaviour as a biomonitor of estuarine health status assessment, as previously suggested by Roast et al. (2001). This integrated methodology should be included in biomonitoring surveys to evaluate habitat vulnerability and potential risk on higher trophic levels.

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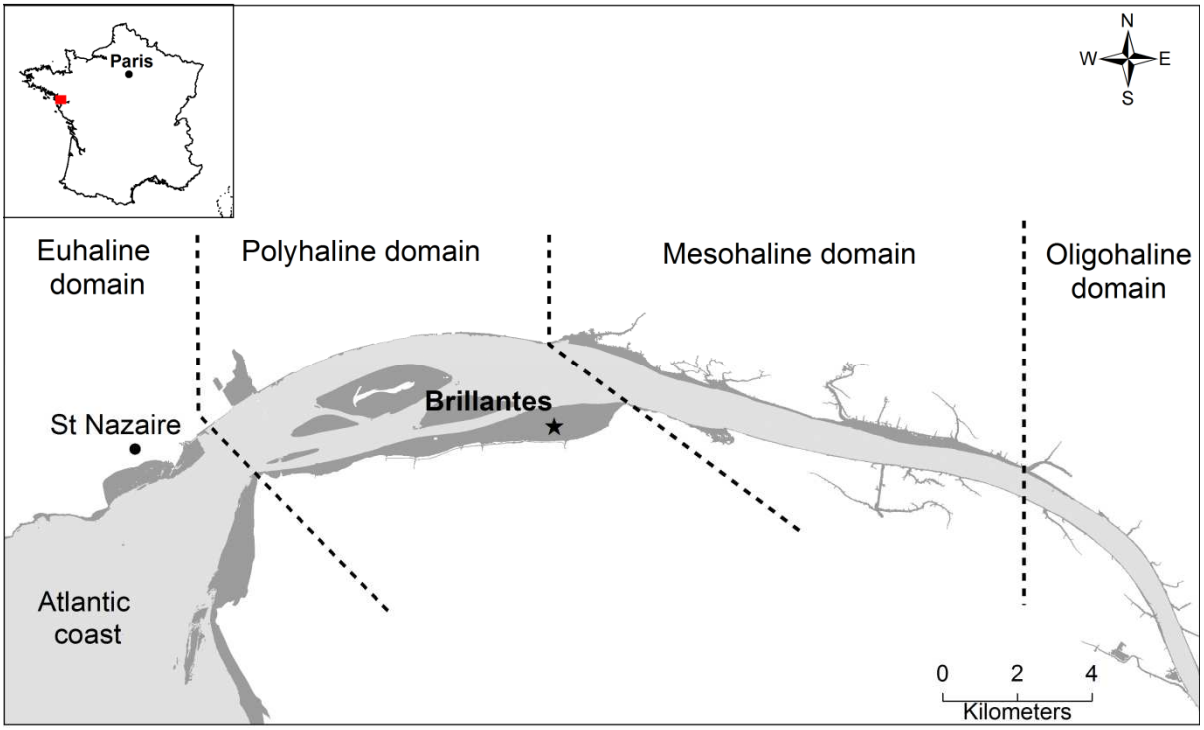

Fig. 1. Brillantes mudflat is localized in the polyhaline domain of the Loire estuary (France), with its intertidal flats shown in dark gray (Benyoucef et al., 2014).

Table 1: AChE activity measured in *H. diversicolor* from site 1 and site 2 expressed in nmole.min^{-1}.mg^{-1} proteins. [a] significantly difference compared to [b] ($p<0.05$).

	AChE activity (nmole.min^{-1}.mg^{-1} proteins) Site1	AChE activity (nmole.min^{-1}.mg^{-1} proteins) Site2	P value
May 2013	19.75±5.01 [a]	11.83±5.70 [b]	0.008
February 2014	30.64±12.42 [a]	6.54±4.61 [b]	0.002

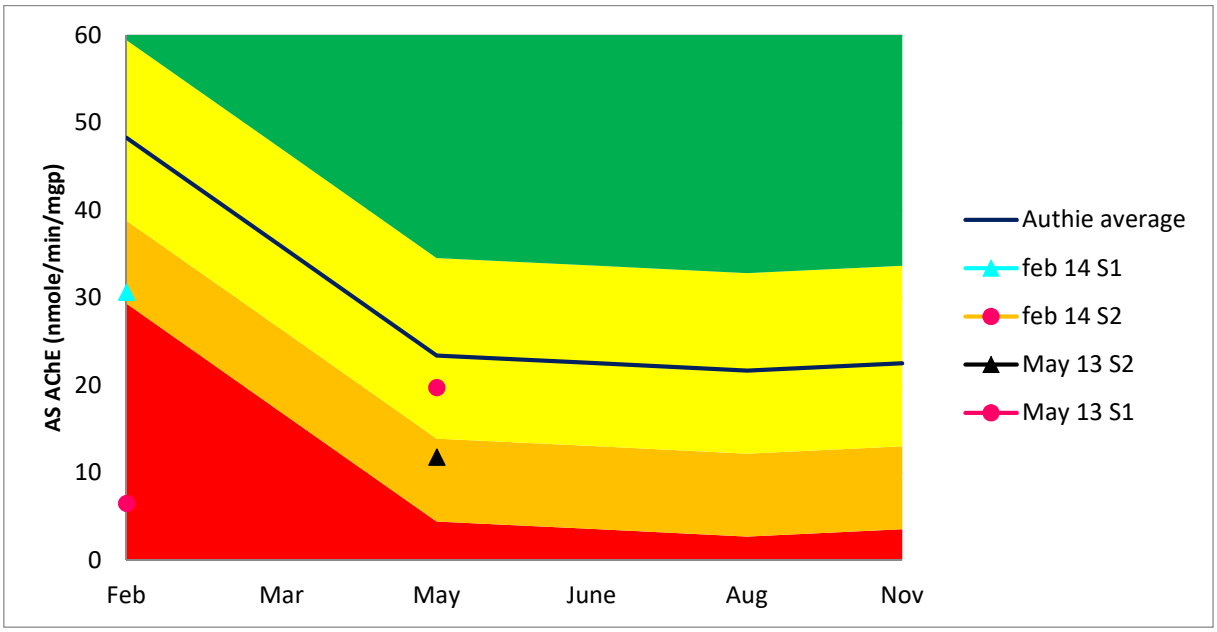

Fig. 2 BAC (baseline assessment criteria) and EAC (environmental assessment criteria) graph for AChE activity values (Barrick et al., 2018). The black line indicates the mean values established for the low-impact site Authie, the yellow band indicates the range of the BAC and the orange line indicates the range where sublethal effects are expected to occur. AChE values for both sites (Site 1: S1; Site 2: S2) on May 2013 (May 13) and February 2014 (Feb 14) were plotted on the graph as well.

Table 2: Burrowing tests: Mean estimate α (h^{-1}) and the 95% credibility interval (CI) obtained after *a posterior* distribution for each site (site 1: S1, site 2: S2) and sampling period (May 2013 and February 2014). MFB: Mean frequency occurrence (%) and standard deviation (SD) of signals recorded with Multi Freshwater Biomonitor (MFB) and corresponding to body undulation (0.5 Hz) and to head movements and feeding

445 activity (1 to 2 Hz) in *H. diversicolor*. * significant difference ($p<0.05$). nd: not
446 determined.

	Burrowing test α [CI] S1	Burrowing test α [CI] S2	MFB Frequency occurrence (mean%±SD) 0.5 Hz S1	MFB Frequency occurrence (mean%±SD) 0.5 Hz S2	MFB Frequency occurrence (mean%±SD) 1.2 Hz S1	MFB Frequency occurrence (mean%±SD) 1.2 Hz S2
May 2013	0.064 [0.036 - 0.099]	0.059 [0.033 - 0.091]	64.89±9.13*	43.95±16.15 * (p=0.021)	10.48±5.88	7.27±2.01 (p=0.523)
February 2014	0.103 [0.063 - 0.152]*	0.036 [0.020 - 0.059]*	58.00±4.43	49.51±7.70 (p=0.066)	6.44±0.97	5.61±0.92 (p=0.173)

447

448 Table 3. Intersite variations of *H. diversicolor* density (number of individuals N m^{-2} as
449 mean±standard deviation) and biomass (Fresh weight FWg m^{-2} as mean±standard
450 deviation) for each sampling period (May 2013 and February 2014). nd: not determined

	Density ($N.m^{-2}$) Site 1	Density ($N.m^{-2}$) Site 2	Biomass ($FWg.m^{-2}$) Site 1	Biomass ($FWg.m^{-2}$) Site 2
May 2013	631.38±182.03	51.02±31.24	39.80±21.13	3.18±1.96
February 2014	280.61±325.69	66.96±28.28	19.84±23.26	4.46±4.64

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