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Multiple-stressor effects on stream invertebrates: DNA barcoding reveals contrasting responses of cryptic mayfly species

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ABSTRACT

Most freshwater ecosystems are subject to multiple anthropogenic stressors, which commonly reduce biodiversity across all levels. Existing freshwater bioassessment programmes aim at identifying responses of aquatic biota to stressors. For practical reasons, higher-level taxonomic groups (e.g. genus or family) are often used in these programmes. This approach, however, may bias assessment results as different species can differ substantially in their biological traits, thus emphasising the need for species-level data. DNA barcoding can reliably generate species-level data for animals by sequencing a fragment of the mitochondrial cytochrome *c* oxidase subunit I gene (*COI*). This allows investigating species-specific responses to environmental stressors. In this study, we sampled 43 stream sites in southern New Zealand spanning wide gradients of agricultural stressors (fine sediment and nutrient levels). We first used conventional morphological assessment to determine stream invertebrate responses to the stressors, focusing on two important indicator taxa, the mayfly *Deleatidium* and the snail *Potamopyrgus*. We then tested for the presence of cryptic species in *Deleatidium* and *Potamopyrgus* using DNA barcoding of the *COI* gene for 520 and 305 specimens, respectively. While all *Potamopyrgus* specimens belonged to a single species, *Deleatidium* consisted of 12 distinct molecularly identified clades that likely represent distinct species. Finally, we compared stressor responses assessed at genus and species level. While overall *Deleatidium* abundance was unrelated to stressor levels, some of the individual clades differed clearly in the magnitude and direction of their responses to nutrient and sediment stress. While the most abundant cryptic *Deleatidium* clade (clade 1) showed no relationship to sediment or nutrient levels, clades 2 and 3 responded negatively to nutrient or sediment increases, respectively. These contrasting patterns indicate that individual freshwater invertebrate species, often merged to a higher taxonomic level for biomonitoring purposes, can differ substantially in their tolerance to stressors and respond in more complex ways than observed at genus level. Overall, our results highlight the considerable potential and importance of including DNA barcoding into freshwater ecosystem assessment and biomonitoring programmes.

1. Introduction

Freshwater ecosystems are hotspots of biodiversity (Dudgeon et al., 2006; Strayer and Dudgeon, 2010). Although only 0.008 percent of the Earth's water is non-saline freshwater in rivers, lakes and swamps (Gleick, 1993), a large part of global biodiversity is directly dependent on this resource. Many freshwater ecosystems, however, are subject to multiple anthropogenic stressors that negatively affect biodiversity across all levels. In particular, the ongoing intensification of agricultural land-use is one of the greatest threats to freshwater biodiversity and associated ecosystem services worldwide (Vörösmarty et al., 2010). To understand and counteract ecosystem degradation, many countries have initiated large biomonitoring programmes that include the regular assessment of water and ecosystem quality by analysing the composition of the macrozoobenthic community, with particular focus on certain indicator taxa (Rosenberg and Resh, 1993; Wood et al., 2013). The data produced by such biomonitoring programmes are important sources for identifying the impacts of single and multiple stressors on freshwater ecosystems (Stendera et al., 2012) and

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assessing the success of restoration programmes (Winking et al., 2014).

In most such bioassessments, the organisms found are determined at higher taxonomic levels than species, i.e. to genus, family or even order. The main reason for this is that identification of benthic freshwater invertebrates at species level is extremely time-consuming and costly (Marshall et al., 2006). Moreover, species-level identifications are often highly unreliable due to misidentifications (Haase et al., 2006, 2010). While higher-level taxonomy can often contain sufficiently robust information for bioassessment (Lenat, 1988; Defeo and Lercari, 2004; Kallimanis et al., 2012; Mueller et al., 2013), species-level data are beneficial as it has been shown repeatedly that even closely related species can have very different tolerances to stressors and different ecological functions (Cranston, 1990; Feckler et al., 2012; Schmidt-Kloiber and Nijboer, 2004). However, several freshwater taxa simply lack morphological diagnostic characters at the larval and even at the adult stage (so-called "cryptic species"; Cook et al., 2008; Liu et al., 2003; Weiss et al., 2014). Therefore, even expert taxonomists cannot identify these species. Similar to non-cryptic species, these cryptic taxa can differ markedly in their ecological requirements and tolerances (Feckler et al., 2012; Fišer et al., 2015; Obertegger et al., 2014; Soucek et al., 2013; Wellborn and Cothran, 2007). This fact adds more weight to the argument that environmental assessments based on higher-level taxonomy are prone to errors (Schmidt-Kloiber and Nijboer, 2004).

Molecular approaches can overcome the central problem of morphological species identification and delimitation of cryptic species, leading to higher taxonomic resolution in ecosystem assessments (Jackson et al., 2014; Sweeney et al., 2011). Furthermore, such genetic data can also provide information on intraspecific genetic diversity and therefore allows assessing a population's vulnerability and adaptability under stressed conditions. For animals, sequencing of a fragment of the mitochondrial cytochrome *c* oxidase subunit I (*COI*) has proven to be highly successful for species identification ("DNA Barcoding", Hebert et al., 2003). This gene is broadly used by different consortia to catalogue the diversity of life (Ratnasingham and Hebert, 2007). Recently, it has also been proposed and tested for use in freshwater biomonitoring programmes (Hajibabaei et al., 2011; Thomsen et al., 2012) and conservation (Sivaramakrishnan et al., 2014). Some recent studies on freshwater ecosystem quality and health, implementing molecular approaches, suggest that species identification using DNA barcodes may allow assessing biodiversity and degradation of freshwater ecosystems in greater detail than classical approaches (Elbrecht and Leese, 2015; Jackson et al., 2014; Pilgrim et al., 2011; Stein et al., 2013).

In this study, we compared stress–response patterns of macroinvertebrate bioindicator taxa using traditional morphology-based metrics that only allow identification at genus level with DNA-based methods. To address this aim, we conducted a large stream survey in the intensely farmed Southland region of New Zealand. We re-sampled 43 stream/river sites previously surveyed by Wagenhoff et al. (2011) who assessed invertebrate responses to two important agricultural stressors, fine sediment and nutrients, based on community-level metrics and traditional assessment methods. Intensive dairy farming in Southland has increased considerably during the last few decades (Drewry and Paton, 2000; Foote et al., 2015), resulting in elevated levels of deposited fine sediment and dissolved nutrients in many streams and rivers (Monaghan et al., 2007; Williamson et al., 1992). These stressors have been shown to negatively affect invertebrate communities in streams worldwide (Allan, 2004; Ramezani et al., 2014; Ryan, 1991; Wagenhoff et al., 2011) and are expected to become an increasingly serious threat to stream ecosystem health in combination with global climate change (Piggott et al., 2015a,b,c).

Our survey design covered a gradient from relatively pristine to heavily degraded streams. We focused on two key indicator taxa in New Zealand streams and rivers, the mayfly *Deleatidium* and the mudsnail *Potamopyrgus*. Mayfly larvae of the genus *Deleatidium* are among the most abundant and widespread stream invertebrates in New Zealand, and the genus is regarded as an important indicator taxon for good ecological water quality (Stark and Maxted, 2007). Sixteen species have been described, but the distribution range of many species is unknown and identification of both adults and larvae is challenging or impossible (Hitchings, 2010). Snails of the genus *Potamopyrgus* are equally abundant and regarded as good indicator organisms for organic pollution (Stark and Maxted, 2007). Both taxa are known to consist of several morphologically cryptic species (Haase, 2008; Hitchings, 2010, 2008; Towns and Peters, 1996), a common phenomenon in mayflies (Rutschmann et al., 2014; Ståhls and Savolainen, 2008; Williams et al., 2006) and snails (Pfenninger et al., 2003; Weigand et al., 2011; Wilke and Pfenninger, 2002). Here, we analysed the mitochondrial COI gene in the two indicator taxa to test two hypotheses: (1) Both *Deleatidium* and *Potamopyrgus* sampled in the survey consist of several divergent clades; (2) The divergent clades unveiled by DNA barcoding differ in their ecological preferences and stress responses and, consequently, the responses observed at higher taxonomic levels may not be representative of individual clade or species-level responses.

2. Materials and methods

2.1. Field sampling

We sampled 43 stream sites (see Wagenhoff et al., 2011 for a detailed description) in the Southland province of New Zealand during a two-week period in April 2012. Stream order ranged from 2nd to 6th order and sampled stream reach length from 5 to 50 m. Stream flows at all sites were largely stable for at least three weeks prior to the survey commencing and during the survey (Environment Southland 2012). Sampling sites were chosen to span the full range of agricultural land use intensities in the province (see Wagenhoff et al., 2011). At each site, the percentage cover of deposited fine sediment (<2 mm) on the stream bed was estimated visually using an underwater viewer following the protocol described in Clapcott et al. (2011). Concentrations of total nitrogen and total phosphorus, dissolved ammonium and nitrate (summed as dissolved inorganic nitrogen, DIN) and dissolved reactive phosphorus (DRP) were determined from four replicate water samples per site following standard methods (Cleceri et al., 1998). The amount of suspendable inorganic fine sediment (SIS) was determined from five randomly collected samples per site using the Quorer method (Clapcott et al., 2011; Quinn and Cooper, 1997).

Invertebrate samples were taken following a standard semi-quantitative kick-sampling method (Stark et al., 2001) using a D-shaped hand net (0.5 mm mesh). At each site, the streambed substratum was disturbed in ten locations of varying flow velocity via kicking for 30 s, thus standardising sampling effort per site. Samples were preserved in 70% ethanol, transferred to 96% ethanol after 2–6 days and stored at 4 °C until further analysis.

In the laboratory, samples were divided into eight equal portions using a rotating subsampler (Waters, 1969). A total of 300 invertebrates per sample were counted and identified to the lowest practical taxonomic level under a stereomicroscope (magnification 8–40×; Olympus SZ51, Olympus Corp., Japan). Further, entire samples were scanned for rare taxa and these were added to the combined number of taxa in the pollution-sensitive orders Ephemeroptera, Plecoptera and Trichoptera (EPT taxon richness) (Stark et al., 2001). The absolute abundances of *Deleatidium* spp.

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