Title: Cumulative effects of multiple stressors: an invasive oyster and nutrient enrichment reduce subsequent invasive barnacle recruitment

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Abstract

Studies identifying interactions between biological invasions and other stressors have generally focussed on quantifying their cumulative effects on mature species assemblages. In benthic systems, however, early life history processes are key determinants of assemblage structure and functioning. This study tested whether the presence of an invasive species affected early life history processes of two common barnacle species and whether this was affected by a second common stressor, nutrient enrichment. The results of a field experiment identified and characterised the effects of an invasive oyster, *Crassostrea gigas*, on the early life history processes of the two barnacle species under ambient and enriched nutrient conditions. In the presence *C. gigas*, the invasive barnacle *Austrominius modestus*, had a lower recruitment rate, however, there was no effect of the presence of *C. gigas* on native barnacle, *Semibalanus balanoides*, recruitment. Nutrient enrichment also reduced the recruitment rate of *A. modestus*, however, there was no evidence of synergistic or antagonistic interactions between these stressors, indicating their cumulative effects were additive. There was no effect of nutrient enrichment on native barnacle recruitment. Our results show that the presence of an invasive oyster and nutrient enrichment altered the recruitment of another non-native benthic species. These findings emphasise the importance of considering early life history processes when assessing effects of multiple stressors on communities.
1. Introduction

Identifying and quantifying the impacts of multiple anthropogenic stressors, such as invasive species and nutrient enrichment, is a research priority in order to understand and predict potential detrimental effects on ecosystems (Crain et al., 2008; Sutherland et al., 2009; Strayer, 2012). Interactions between invasive species and other anthropogenic stressors can lead to cumulative effects that are additive or are greater than (synergistic) or less than (antagonistic) the sum of the individual effects (Folt et al., 1999; Crain et al., 2008). Synergistic cumulative effects on communities are thought to be the most common (Sala and Knowlton, 2006) and their occurrence has been supported by several empirical studies. For example, Piazzi et al. (2005) showed a decline in percentage cover of erect algal species when exposed to the invasive green algae *Caulerpa racemosa var. cylindracea* in increased sedimentation regimes. Conversely, antagonistic interactions have also been identified, such as the ability of the invasive freshwater zebra mussel, *Dreissena polymorpha*, to negate the effects of nutrient enrichment on algal biomass (Dzialowski and Jessie, 2009), and the presence of *Sargassum muticum*, an invasive fucoid algae, mediating the effects of nutrient enrichment and warming on algal biomass (Vye et al., 2015).

To date, studies have focussed on the context-dependent impacts of biological invasions on the diversity and functioning of mature communities (e.g. Queiros et al., 2011; Green and Crowe, 2014). In benthic ecosystems, the structure and functioning of a mature community can be determined by early life history processes, such as larval settlement and post-settlement mortality (Connell, 1985; Gaines and Roughgarden, 1985; Hunt and Scheibling, 1997; Aguilera and Navarrete, 2012). Settlement, defined as the permanent attachment of larvae to the substratum (Connell, 1985), is often determined by larval supply and a range of settlement cues that indicate habitat suitability and resource availability, such as the presence of free space and biofilm abundance (Strathmann et al., 1981; Rodriguez et al., 1993). Early
post-settlement mortality may be driven by predation, disturbance or physiological stress (Menge and Sutherland, 1987). Both settlement and early post-settlement mortality can constrain recruitment into the adult population and, therefore, are important components of benthic species population dynamics (Gosselin and Qian, 1997; Delany et al., 2003; Jenkins, 2005). The relative importance of these early life history processes in structuring communities can be context specific. Early post-settlement mortality is generally more important in determining population structure in species with high recruitment rates, such as barnacles (Connell, 1961a; Gosselin and Qian, 1996), whereas populations of species with a lower larval supply, such as some species of corals (Hughes et al., 2000), crustaceans (Wahle and Incze, 1997) and echinoderms (Balch and Scheibling, 2000), are more likely to be affected by differences in settlement rates (Connell, 1961a). Invasive species, in combination with other stressors, such as nutrient enrichment or warming, may drive changes in settlement and post-settlement mortality by altering physical conditions, such as substratum type and hydrological regimes, and biological interactions, such as competition and predation (Gutierrez et al., 2003; Wilkie et al., 2013).

In coastal ecosystems, bivalve molluscs are common invasive species. Outside of its native range, the Pacific oyster, Crassostrea gigas, has wide-ranging and context-dependent effects on recipient communities, including driving shifts in native species assemblage structures (Kochmann et al., 2008), differences in ecosystem functioning rates (Green et al., 2012), and the co-introduction and facilitation of other invaders (Ruesink et al., 2005). Often the impacts of C. gigas increase in intensity as invasion progresses and the density of the oysters increases (Yokomizo et al., 2009; Green and Crowe, 2013). Although the impacts of C. gigas on mature communities are well documented (e.g., Padilla, 2010), little is known about the potential interactions between C. gigas and native or invasive species at early life history stages (Wilkie et al., 2012).
C. gigas forms feral populations in inlets and estuaries, where eutrophication is a common co-occurring stressor that can affect the impacts of biological invasions on recipient communities (Lotze et al., 2006; Gennaro and Piazza, 2011; Vaz-Pinto et al., 2013). Thus, testing whether the presence and density of C. gigas interacts with nutrient enrichment to affect settlement and recruitment processes is a realistic scenario from which to identify the context-dependent effects of invasive species. A field experiment was designed to test for the separate and cumulative effects of the presence of C. gigas and nutrient enrichment on benthic species settlement and recruitment rates. Specifically, the hypothesis tested were: (1) the presence of invasive C. gigas and nutrient enrichment will affect the identity and abundance of other benthic species settlers and recruits; (2) these putative effects will interact, such that the effect of the presence of the invasive oyster on other benthic species settlement and recruitment will differ between ambient and enriched nutrient conditions; (3) the cumulative effects of the presence of the invasive oyster and nutrient enrichment on other benthic species will be determined by oyster density.

2. Material and methods

2.1. Study site

The field experiment ran from February through to August 2013 at Ballygreen, a sheltered intertidal sedimentary shore on the south western shore of Lough Swilly, Co. Donegal, Ireland (55° 2’ 31.54” N, 7° 33’ 36.06”W). At this site, boulders are common and scattered on sediment comprised of sandy mud, pebbles and shell fragments. Tides are semi-diurnal and have a maximal range of approximately 4.5 m. The study was conducted at mid shore where boulders were colonised primarily by the native barnacle Semibalanus balanoides and the non-native barnacle Austromininius modestus (formerly Elminius modestus), the fucoid algae Fucus vesiculosus, the honeycomb worm Sabellaria alveolata and the keel worm,
Pomatoceros triqueter. Austrominius modestus has spread rapidly since its introduction to the UK and Ireland in the 1940s and may compete with native barnacle species (Bishop, 1947; Crisp, 1958; Lawson et al., 2004). Lough Swilly is a relatively unpolluted estuary compared to other more densely populated coastal areas of Ireland that have been classified as eutrophic in assessments of water quality (Bradley et al., 2015).

2.2. Experimental design and set up

To quantify benthic species recruitment under manipulated conditions, forty grey opaque Perspex® settlement plates (210 mm x 148 mm x 5 mm) were attached to the side of boulders (one per boulder), which had been selected randomly along approximately 40 m x 10 m of mid shore dominated by barnacles and Fucus vesiculosis. Grey Perspex® was chosen to represent natural conditions based on the colour of the bedrock to minimise any differences in thermal regime between the settlement plates and boulders (Lathlean and Minchinton, 2012). Each plate was sanded for thirty seconds using coarse sand paper to ensure suitable rugosity for settlement (Jara et al., 2006). Plates were attached to boulders at least two metres apart using stainless steel screws (Stachowicz et al., 2002; Canning-Clode et al., 2008).

An orthogonal experimental design included two fixed factors: (i) presence of the invasive C. gigas at four levels: absent, 1 individual (ind.) per plate, 4 ind. or 8 ind. (equivalent to approximately 0, 32, 129, and 515 individuals per m²); and (ii) nutrient enrichment at two levels: ambient conditions and nutrient enriched. Each treatment was replicated five times, yielding 40 experimental units. Settlement plates were allocated randomly to treatments.

Non-reproductive triploid oysters (Guo and Allen, 1994) from a local aquaculture facility were used to minimise effects on the feral oyster population. Juvenile oysters (spat) were used in the experiment and were six months old and 36 ± 0.5mm in length, similar to the age and size of naturally settled spat at the time the experiment commenced. Spat were attached
to the front surface of the settlement plates using Milliput® epoxy putty (Dolgellau, Wales).

Previous work showed that there were no differences in assemblages associated with *C. gigas* attached using this method compared to those with *C. gigas* attached naturally (Vye, unpublished results).

Localised nutrient enrichment was achieved by attaching nutrient diffusers (drilled 50 ml sample tubes) to each plate. Diffusers were filled with 140g of Everris Osmocote® Exact (Geldermalsen, Netherlands) slow release fertilizer pellets (11N:11P:18K) similar to previous studies (e.g., Hall et al., 2000; Minchinton and McKenzie, 2008; O’Connor and Donohue, 2013). Ambient treatments had diffusers filled with shell fragments to limit potential experimental artefacts. Analysis of water samples from within a 15 cm radius of experimental plates 8 weeks after the addition of fertiliser pellets using the same method indicated that nutrient enrichment was effective (ambient total oxidised nitrogen (mean ± S.E.): 10.54 ± 0.81 µm l⁻¹, enriched total oxidised nitrogen: 14.24 ± 1.44 µm l⁻¹, ANOVA: $F_{1, 14} = 5.014$, $P = 0.042$).

The top surface of each plate was monitored every two to four weeks to ensure treatments were maintained and photographed at eight weeks and 24 weeks (Fig. 1). Abundance of all species on each plate was estimated from photos, as the new community was mono-layered and this method was more accurate than estimating percentage cover using grid quadrats and the point intercept method (Foster, 1991; Meese, 1992). During the experiment, the plates were colonised by the native barnacle, *S. balanoides*, and the non-native barnacle, *A. modestus*. A matrix of sediment and juvenile fucoid (< 2cm) was present on four out of the forty plates after twenty four weeks but these plates were distributed evenly among treatments and this was not considered in our analysis. Total abundance of all barnacles and abundance of each species were estimated using the Cell Counter plugin in ImageJ photo processing software (Schneider et al., 2012). At eight weeks, barnacle settlement had
occurred and cyprid larvae and recently metamorphosed juvenile barnacles were present on
the plates. These were grouped under the term ‘settlers’ because it was not possible to
distinguish between cyprid and juvenile barnacles accurately (Caffey, 1985; Jenkins et al.,
1999; O’Riordan et al., 2004; Cruz et al., 2005; Power et al., 2006). It was also not possible
to identify barnacles to species level at 8 weeks and, therefore, total barnacle abundance was
used in analysis. At 24 weeks, barnacles were large enough to distinguish between species,
allowing individual estimates of the abundance and mean size of *S. balanoides* and *A.
modestus* per plate to be quantified. Estimates of percentage cover of each species relative to
the free space available to them were used for the analysis. *S. balanoides* individuals were
larger (mean ± S.E.: 19.13 ± 0.95 mm²) than *A. modestus* (mean ± S.E.: 15.40 ± 0.79 mm²)
and thus occupied a greater area of space than *S. balanoides* even when species abundances
were similar. Focussing on percentage cover of each species, rather than abundance, is
therefore more meaningful when comparing benthic recruitment rates in communities where
settling space is a limiting resource, such as rocky shore communities (Dayton, 1971). In
addition, we tested whether barnacle density differed between oysters shell and experimental
plates to assess whether preferential settlement on oysters occurred. An oyster was selected
haphazardly from plates with *C. gigas* (from treatments with 4 & 8 individuals/ plate) and
barnacle density on the oysters was estimated (individuals cm⁻¹) and compared to barnacle
density on the plates and was shown not to differ significantly (t = 1.279, df = 18, P = 0.216).

2.3. Data analysis

Analysis of variance (ANOVA) was used to test all hypotheses with density of *C. gigas* (four
levels) and nutrient enrichment (two levels) as fixed factors. Data were tested for assumptions
of homogeneity of variances using Levene’s test and normality was examined using Q-Q
plots and Shapiro-Wilk tests. Total abundance of settlers and recruits at 8 weeks and 24
weeks were log transformed. Percentage data were arcsine square root transformed (Sokal
and Rohlf, 1995). Student-Newman-Keuls tests were used to make post-hoc comparisons among levels of significant terms. All statistical analyses were carried out in R 2.15.3 (R Development Core Team, 2011).

3. Results

At eight weeks, there was no significant interaction between the presence of *C. gigas* and nutrient enrichment ($F_{3,31} = 0.773, P = 0.518$, Fig. 2A) on the total abundance of settled barnacles on the experimental plates. The presence of *C. gigas* did not affect the total abundance of settled barnacles on the experimental plates significantly even at the greatest oyster density level ($F_{3,31} = 1.348, P = 0.277$, Fig. 2A). There was no significant effect of nutrient enrichment on the total abundance of settled barnacles ($F_{1,31} = 0.228, P = 0.636$; Fig. 2A). At 24 weeks, there was no significant interaction between the presence of *C. gigas* and nutrient enrichment ($F_{3,31} = 1.719, P = 0.183$, Fig. 2B) on total barnacle abundance. There was a significantly greater total abundance of barnacles on settlement plates where *C. gigas* was absent compared to all treatments with *C. gigas*, regardless of oyster density ($F_{3,31} = 3.279, P = 0.034$, Fig. 2B and Fig. 2B(i)). However, there was no significant effect of nutrient enrichment on total barnacle abundance ($F_{1,31} = 2.104, P = 0.183$, Fig. 2B).

At 24 weeks, when barnacle species could be distinguished, the native *S. balanoides* constituted 26 ± 2 % (mean ± S.E.) of the total abundance of barnacles with the remaining 74 ± 2 % (mean ± S.E.) comprised of the smaller invasive barnacle *A. modestus* across all treatments. Mean total percentage cover of barnacles on the plates was 15.1 ± 2.4 % (± S.E.). There was no significant interaction between the presence of *C. gigas* and nutrient enrichment ($F_{3,31} = 0.793, P = 0.507$, Fig. 3A) on the percentage cover of *S. balanoides*. There was also no significant effect of the presence of *C. gigas* ($F_{3,31} = 1.030, P = 0.393$, Fig. 3A), nor of nutrient enrichment ($F_{1,31} = 0.059, P = 0.810$, Fig. 3A) on percentage cover
of *S. balanoides*. There was no significant interaction between the presence of *C. gigas* and nutrient enrichment \((F_{3,31} = 2.082, P = 0.123)\) on the percentage cover of the invasive barnacle *A. modestus*. Both the presence of *C. gigas* \((F_{3,31} = 3.329, P = 0.032, \text{ Fig. 3B and 3B (i))}\) and nutrient enrichment \((F_{1,31} = 4.374, P = 0.045, \text{ Fig. 3B and 3B (ii))}\) had significant negative effects on the percentage cover of *A. modestus*, however, there was no significant effect of increasing densities of *C. gigas* (Fig. 3B (i)).

4. Discussion

This study tested empirically for effects of invasive species, coupled with nutrient enrichment, on the early life history processes of two species of barnacle and identified negative effects of an invasive species and nutrient enrichment on invasive barnacle recruitment. The effects of both factors on barnacle recruitment were independent of each other indicating that the cumulative effect of both *C. gigas* presence and nutrient enrichment were additive. These effects on barnacle recruitment, however, were not consistent across both species, affecting an invasive but not a native species. Recruitment of the invasive barnacle, *Austrominius modestus*, was lower in the presence of the invasive oyster, *Crassostrea gigas*, whereas recruitment of the native species, *Semibalanus balanoides* was not affected by either stressor. Furthermore, increasing the density of *C. gigas* did not enhance their negative effect on recruitment of *A. modestus*, indicating that this effect was not density-dependent, which shows that even at low densities the presence of an invasive species can determine subsequent community dynamics. These findings also show that the effects of an invasive species on other benthic species recruitment varies between different species of recruits and are not determined necessarily by the presence of a secondary stressor, such as nutrient enrichment, or the density of the invasive species.
The negative effect of the presence of *C. gigas* on recruitment of the invader *A. modestus* may have been driven by reduced settlement or increased post-settlement mortality rates. Differential settlement may have occurred, where all the settlers at 8 weeks, which could not be identified to species level, were the native barnacle, *S. balanoides. Semibalanus balanoides* showed no response to *C. gigas* or nutrient enrichment treatments at 24 weeks, indicating that *S. balanoides* settlement and recruitment may not be affected by these treatments. Hence, the effects seen at 24 weeks may have been a result of the subsequent reduced settlement and recruitment of *A. modestus* between the two sampling events.

Differential settlement may occur where there are inter-species differences in larval supply or settlement cues (Bohn et al., 2013). Alternatively, if differential settlement did not occur, treatment effects could have been on post-settlement mortality rather than reduced settlement because the effects were detected only at 24 weeks. It is possible that there was a density-dependent reduction in settlers after eight weeks, as the presence of *C. gigas* reduced the free space available for settlement. This is not probable, however, because of the known gregarious behaviour of barnacles (Barnett and Crisp, 1979) and the relatively large amount of free space (approximately 60%) remaining on the experimental plates. The similar density of barnacles on settlement plates compared to *C. gigas* shells also indicates that no preferential settlement occurred on the oysters that may have confounded any effects of *C. gigas* on settlement. These findings suggest that effects of the presence of the invasive oyster could have manifested at post-settlement mortality stages. Post-settlement mortality is a key determinant of population dynamics in barnacles and, thus, the effects of *C. gigas* and nutrient enrichment on barnacle early life history may propagate through time to impact the diversity and functioning of mature benthic communities (Hunt and Scheibling, 1997; Delany et al., 2003).
The lower total barnacle recruitment rate, primarily a result of fewer *A. modestus*, may have been caused by a range of mechanisms. According to optimal foraging theory (MacArthur and Pianka, 1966), the addition of *C. gigas* may represent an increase in prey resource (Pyke, 1984) for consumer species, such as the common shore crab *Carcinus maenas*, that may prey upon both juvenile oysters and barnacles (Diederich, 2005). This may lead to enhanced predation levels on the settlement plates. Alternatively, the physical presence of *C. gigas* may have led to increased turbulence around the plate, increasing mortality directly owing to physical disturbance (Crimaldi et al., 2002; Gutierrez et al., 2003). In addition, *C. gigas* could affect mortality indirectly by mechanisms including filter feeding, which would reduce food supply, and by causing differences in biofilm composition by altering hydrology (Thompson et al., 2005; Neal and Yule, 2009). In each of these potential mechanisms, a density-dependent effect of *C. gigas* may have been expected, however, we did not identify any density-dependence in this study. This may have been because *C. gigas* covered only approximately 45% of each plate at the highest density, which may have not been sufficient for density effects on recruitment to become apparent (Wagner et al., 2012; Wilkie et al., 2013). It has been hypothesised that the presence of invasive species may affect the recruitment of other non-native species, either by increasing non-native species recruitment under the invasional meltdown hypothesis (Simberloff and Holle, 1999), or by reducing non-native species recruitment by increasing community invasion resistance (Elton, 1958; Balmford, 1996; Levine and D’Antonio, 1999). Our results show that the recruitment rate of the non-native barnacle, *A. modestus* was lower when *C. gigas* was present, which is not consistent with the invasional meltdown hypothesis. Invasional meltdown occurs when there are facilitative direct or indirect interspecific interactions amongst invasive species (Simberloff and Holle, 1999), suggesting that in this study there were no facilitative interactions between *A. modestus* and *C. gigas*. 
Nutrient enrichment also decreased the percentage cover of *A. modestus*, which again was probably driven by increasing post-settlement mortality. Direct effects of nutrient enrichment on post-settlement mortality of *A. modestus* may have been caused by increases in ammonia concentrations within nutrient enriched treatments, which has been shown previously to affect recruitment in benthic invertebrates (Fitt and Coon, 1992), or as a result of other compounds, such as potassium (Kang et al., 2004), incidentally released in nutrient enriched treatments (Pawlik and Hadfield, 1990; Pawlik, 1992; Minchinton and McKenzie, 2008). Nutrient enrichment may also have reduced recruitment by causing differences in the abundance and composition of biofilm, an important food resource for intertidal grazers (Jenkins et al., 2001; Hill and Hawkins, 2009), and therefore, increased grazing rates on the settlement plates (Thompson et al., 2000). Grazing activity by the limpet, *Patella vulgata* (Lewis, 1954) and the periwinkle *Littorina littorea* (Connell, 1961a; Dayton, 1971), both present at the study site, have been linked to increased biological disturbance and, thus, increased post-settlement mortality of newly settled cyprid larvae and juvenile barnacles (Lewis, 1954; Connell, 1961b; Dayton, 1971; O’Connor et al., 2011). Our results are contrary to studies in other systems that have found nutrient enrichment to increase invasion (Bertocci et al., 2015; Gennaro and Piazzi, 2011), suggesting the impacts of nutrient enrichment on invasive species are likely determined by the main life-history traits of the species examined. Despite the predicted widespread occurrence of synergistic and antagonistic cumulative impacts of multiple stressors (Sala and Knowlton, 2006; Crain et al., 2008), this study found only additive cumulative effects of the presence of *C. gigas* and nutrient enrichment on *A. modestus* recruitment. Additive effects are estimated to occur in approximately 25% of multiple stressor scenarios (Crain et al., 2008) and may allow greater predictability of cumulative effects where there is sufficient information describing the direct effects (e.g. Chiu et al., 2008; Rius et al., 2009). The additive cumulative effects were identified over a
relatively short time period in this study, however, the nature of the interaction among
stressors may shift over longer time periods as the effects of the stressors develop (Darling
and Côté, 2008). In light of this, continued environmental change, such as ocean warming
and changes in climate variability, during community development over a longer time scale
may lead to more indirect and unpredictable impacts on communities and their functioning
(Crain et al., 2008).

We have shown that the additive cumulative effects of species invasion and nutrient
enrichment differed between recruiting species and, thus, have potential consequences for
population dynamics and the assemblage structure of mature communities. This study
highlights the importance of considering the effects of invasion, in combination with other
anthropogenic stressors, on processes and events across a range of life history stages in order
to fully comprehend multiple stressor impacts on communities. Future work should focus on
determining the mechanisms causing the individual and cumulative effects of invasion and
nutrient enrichment on recruitment using natural substrata, more complex communities and
over longer time periods. We should aim to identify the specific contexts at different life
history stages that determine interactions among multiple stressors in order to advance our
understanding of multiple stressor impacts.

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References


Figure legends

Fig. 1: Experimental plate showing settlement and recruitment of barnacles after 24 weeks.

Fig. 2: Mean abundance of barnacle settlers (± S.E.) per plate (31.1 cm²) with absence and increasing densities of *C. gigas* at eight weeks (A) and 24 weeks (B). Open bars represent ambient nutrient treatments and closed bars are enriched nutrient treatments. Fig. 2B (i) means of *C. gigas* density treatments across ambient and nutrient enriched conditions based on SNK tests. Significant differences among means are indicated by different lower case letters (*P* < 0.05).

Fig. 3: Mean percentage cover (± S.E.) of *S. balanoides* (A) and *A. modestus* (B), at 24 weeks. Fig. 3B (i) means of *C. gigas* treatments across ambient and nutrient enriched conditions and Fig. 3B (ii) means of nutrient enrichment treatments across *C. gigas* treatments. Open bars and ‘A’ represent ambient treatments and closed bars and ‘N+’ represent nutrient enriched treatments. Significant differences among treatments or levels of treatments are indicated by lower case letters (*P* < 0.05).