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Lowe *et al.* Isolation and culturing of *O. marina*

Collection, isolation, and culturing strategies for *Oxyrrhis marina*

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ABSTRACT

The heterotrophic marine flagellate *Oxyrrhis marina*, is a popular and tractable model organism, being common in the intertidal environment and relatively straight forward to maintain in the laboratory. In this report, based on our experience of collecting and culturing ~400 isolates, we provide a quantification of how ‘easy’ *O. marina* is to locate and isolate from the environment and subsequently maintain in culture. In addition, we provide a brief review of the literature pertaining to *O. marina* culturing, stressing in particular the broad physiological tolerance and wide diet range of this organism. Finally, in light of the increasing interest in the genetics and genomics of *O. marina*, and heterotrophic protists more generally, we highlight existing strategies for culturing heterotrophs for ‘-omic’ applications, and summarise our specific culturing approaches, for genomic and proteomic studies of *O. marina*. In this context, we outline a simplified version of Droop’s medium for axenic culturing and offer details on the use of heat-killed *Escherichia coli* as a simple and robust food source for culturing *O. marina* in the absence of other eukaryotes and live prokaryotes.

Key Words: Model protist, Axenic culturing, Intraspecific variation

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INTRODUCTION

Protist experimental models should be robust and, therefore, easy to culture. Furthermore, they should be abundant and easy to isolate from the environment, such that experimental observations can be compared between replicate isolates. Finally, model organisms should be interesting and relevant tools to address fundamental biological, ecological and evolutionary questions (Montagnes *et al.*, this issue).

The heterotrophic marine flagellate, *Oxyrrhis marina*, has proved to be a popular model organism, as demonstrated by the broad scope of the literature concerning this protist (e.g. Flynn and Davidson 1993; Buskey *et al.* 1998; Lowe *et al.* 2005,2010; Saldarriaga *et al.* 2003, Slamovits *et al.* 2007). It is also apparent that *O. marina* is a tractable ‘lab rat’. Indeed, two commonly stated anecdotes about this species are that it is easy to grow and it is abundant in the environment. Striking features from the literature are that *O. marina* has broad environmental tolerances (e.g. Droop 1959; Lowe *et al.* 2005), and an exceptional range of prey types support its growth (see Supplementary Table 1 and references therein). Nevertheless, despite the common use of *O. marina* in laboratory studies, explicit reports of culturing strategies and growth requirements are scarce, with the exception of Droop’s studies (Droop, 1959a, b; Droop and Doyle, 1966; Droop and Pennock, 1971) and other brief reports scattered throughout the literature. A reassessment and synthesis of methods associated with *O. marina* culturing is, therefore, timely.

The second observation, that *O. marina* is abundant and easy to locate in the environment, is certainly anecdotal, as explicit studies examining its abundance and distribution are rare. *Oxyrrhis marina* is undoubtedly widespread: ~50-80 isolates are reported in the literature from a broad range of locations (Lowe *et al.*, 2010). However, estimates of the effort required to obtain isolates from the environment are absent from the literature. Given that recent studies on *O. marina* indicate extensive genetic and phenotypic

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diversity, which may be spatially structured (Lowe *et al.* 2005, 2010), the isolation and characterisation of further strains is likely to be an important component of studies concerning this model protist. Indeed, it is now clear that there is a conspicuous lack of understanding of population level demographic processes for free-living protists in general (Martiny *et al.* 2006), and that in a broader microbial context free-living protists remain relatively poorly described, particularly in terms of population genetic and genomic structure (Caron *et al.* 2009). In this context, sampling guidelines to aid researchers to collect new *O. marina* strains (e.g. to allow intraspecific comparative studies) will be useful.

Here, we examine the literature on *O. marina* pertaining to isolating, culturing, and maintaining isolates. First, we outline our experiences of isolating and establishing *O. marina* cultures; based on the collection of >1000 environmental water samples, we provide estimates of the rate of recovery of *O. marina* from the environment and provide a guide to how readily cultures can be established from such samples. We then highlight common culturing strategies for the maintenance of *O. marina*, and emphasize its wide physiological tolerances and the breadth of prey items that support growth. Finally, in light of the increasing interest in the genomics and proteomics of free-living protists in general (Caron *et al.* 2009), including specific targeted studies of *O. marina* (e.g. Slamovits *et al.* 2007; Slamovits and Keeling 2008), we consider the culturing requirements of such applications and outline three strategies for generating axenic cultures and ‘clean’ material for *de novo* genomic and proteomic analyses. In particular, we revisit Droop’s work on osmotrophy and provided a simplified version of his fully derived medium (Droop and Pennock, 1971) for axenic culturing of *O. marina*.

COLLECTION AND ISOLATION OF *O. MARINA* FROM THE ENVIRONMENT

Recovery of *O. marina* from coastal habitats

1 Lowe *et al.* Isolation and culturing of *O. marina*

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3 *Oxyrrhis marina* is commonly described as cosmopolitan and abundant in coastal habitats;
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5 the interested reader is directed to Watts *et al.* (this issue) for an analysis of *O. marina*
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8 distributions. Most reports describe *O. marina* from coastal environments, particularly supra-
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10 littoral tide pools on rocky shores (e.g. Droop, 1953; Jonsson, 1994; Johnson, 2000), salt
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12 marshes, estuaries (Johnson *et al.*, 2003), and coastal embayments (Begun *et al.*, 2004);
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14 though some of the earliest reviews of the taxon described *O. marina* as most commonly
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16 occurring in saltwater aquaria (Kofoid and Swezy 1921). These observations are for the most
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18 part anecdotal; indeed explicit studies on the abundance and distribution of *O. marina* are
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20 relatively rare and limited in scope (though see Johnson, 2000; Lowe *et al.*, 2010). Thus,
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22 whether our current understanding of distributions and abundance is driven by sampling bias
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24 or is a genuine reflection of *O. marina* distribution in the environment is difficult to
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26 determine.
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32 Nevertheless, the frequency with which *O. marina* has been collected from coastal
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34 habitats certainly suggests abundance: ~40 isolates are described in the literature (see Lowe
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36 *et al.*, this issue), and a further 38 were detailed in a recent study by Lowe *et al.* (2010) – all
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38 of these were isolated from coastal, and almost exclusively intertidal, habitats. However, a
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40 quantification of how easy, or not, *O. marina* is to recover from such habitats is absent from
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42 the literature. As part of an ongoing three-year study into the distribution of *O. marina*, we
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44 have undertaken an extensive sampling program, focusing primarily on northern European
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46 and Mediterranean waters, but also collecting globally. Currently, ~1000 environmental
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48 samples have been collected (Watts *et al.*, this issue, describe the broad trends in the
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50 distribution of *O. marina* in these samples), and our subsequent sampling database provides a
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52 number of useful guidelines for the collection of *O. marina*.
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58 First, the rate of recovery of *O. marina* from intertidal environments is remarkably
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60 consistent; across our whole study, *O. marina* is consistently recovered from ~50% of

Lowe *et al.* Isolation and culturing of *O. marina*

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3 intertidal samples (Fig. 1a). Second, sampling effort has a clear affect on the recovery *O.*
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6 *marina* from a given location (Fig. 1b). Typically, we collect between one and ten 50 ml
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8 samples from a location, though up to 70 samples have been collected from a single site, and
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10 160 samples were collected at one time from a 2 km shoreline in North Wales, UK. Where a
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12 single sample is collected from a given intertidal location (e.g. a stretch of shoreline), there is
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14 on average a ~40% chance of finding *O. marina*; the probability of collecting at least one
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16 positive sample increases to 90-100% if >10 samples are collected from one shore (e.g.
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18 samples from different tide pools; Fig. 1c). Of course, these figures are estimates and depend
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20 on the habitats sampled. Predominately, our samples have been collected from littoral pools
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22 on rocky shores, though we have also isolated *O. marina* from estuaries, salt marshes, coastal
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24 lagoons, and marinas. Nevertheless, a summary of our sampling data provides a useful
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26 guideline for researchers wanting to isolate *O. marina*. Our recommendation is that the
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28 collection of 10-20 50-ml samples from an intertidal habitat (especially a rocky shore with
29
30 littoral pools) will provide a high probability of obtaining *O. marina*.
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36 Finally, seasonality in *O. marina* abundance undoubtedly occurs. Periodic sampling
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38 of a tide-pool at Port St. Mary, Isle of Man revealed peaks in abundance in May-June then
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40 late September-October, interrupted by periods of scarcity in midsummer and winter
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42 (Kimmance, 2001; but also see Fig. 4 in Watts *et al.*, this issue). We have predominately
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44 sampled in the northern hemisphere during the spring and summer; nevertheless, samples
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46 taken during the winter months (when *O. marina* abundance is relatively low) have also
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48 yielded isolates; thus, it can be obtained from the environment throughout the year (Fig. 1b).
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54 55 **Isolation and establishment of clonal cultures**

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57 The above observations support the notion that *O. marina* is relatively straightforward to
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59 locate in coastal habitats. Once collected, cultures are also simple to establish, as *O. marina*
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1 Lowe *et al.* Isolation and culturing of *O. marina*

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3 is large enough (15-40 μm long, Fig. 2) to be manipulated using finely drawn Pasteur
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5 pipettes. Common single cell isolation strategies can then be used to generate clonal cultures
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8 (*e.g.* Stein, 1973; Kemp *et al.*, 1993). In our laboratory, we typically screen environmental
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10 samples using stereo dissection microscopes (Fig. 2); for a description of the gross
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12 morphology of *O. marina* see Lowe *et al.* (this issue). When *O. marina* is detected,
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14 individual cells are transferred to single wells in 24-well (2 ml) tissue culture plates with $\sim 5 \times$
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16 10^4 cells ml^{-1} of the autotrophic flagellate *Dunaliella primolecta* as food. In cases where *O.*
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18 *marina* is not immediately detected, environmental samples are inoculated with *D.*
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20 *primolecta* and re-screened 7 to 14 d later (*i.e.* *O. marina* initially present in samples at low
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22 concentrations may be undetected – an incubation period with food often allows cells to
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24 increase to detectable levels; note though that any mesozooplankton present in samples
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26 should be removed as they graze both *O. marina* and their prey). Monoclonal cultures can be
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28 established following three or four rounds of single cell isolation over a period of two to four
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30 weeks. In our experience, on average, 40% of isolated cells divide successful and generate
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32 moderately dense ($\sim 10^3$ cells ml^{-1}) cultures– though in a small number of cases all single cell
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34 isolations from a sample may die (Fig. 1d).
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44 OXYRRHIS AS A LABORATORY ORGANISM

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46 Undoubtedly, *O. marina* has become a useful experimental model organism because it is
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48 relatively easy to isolate from the environment (as highlighted above) and simple to maintain
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50 in the laboratory. This robustness is a result of broad tolerances to a range of environmental
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52 variables and an exceptionally wide diet range (Supplementary Table 1) – including the
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54 ability to maintain population growth on a fully derived cell-free medium (Droop and
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56 Pennock 1971). Below we summarise our strategy for the maintenance of *O. marina* stock
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58 cultures, as an indication of basic culturing requirements (at the time of writing, we maintain
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~150 isolates in our laboratory; subcultures may be obtained by contacting the corresponding author). We then briefly examine the breadth of physiological tolerances in *O. marina* and assess its nutritional requirements.

Routine maintenance

Once established, *O. marina* is straightforward to maintain in the laboratory. We follow culturing methods described in much of the literature for growing temperate marine protozoa (see Kemp *et al.*, 1993). Briefly, stock cultures of autotrophic flagellates (e.g. *D. primolecta*; see Nutritional requirements, below) obtained from a commercial culture collection (e.g. Culture Collection of Algae and Protozoa, Dunstaffnage, Scotland) are grown in sterilised 32 PSU artificial seawater or filtered natural seawater; both water types are enriched with f/2 medium (Guillard, 1975 - commercially available from Sigma). We do not generally attempt to make our stock cultures of prey or *O. marina* bacteria free, and undoubtedly *O. marina* augments its diet with these bacteria (Jeong *et al.*, 2008). Both prey and *O. marina* cultures are exposed to 24 h irradiance, at $\sim 50 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$, at 15-20 °C; note though that light cycle and temperature will alter growth rates (e.g. Jakobsen and Strom, 2004; Kimmance *et al.*, 2006). *Oxyrrhis marina* is maintained on prey flagellates at $\sim 10^5$ cells ml⁻¹. Typically, cultures are kept in ~ 75 ml flasks and transferred every four weeks. Using this strategy we have maintained a collection of 150-200 stock cultures for >12 months with a $\sim 5\%$ failure rate (i.e. 5% of the stock cultures have died).

Physiological tolerance

Oxyrrhis marina is robust to a number of parameters that may change in culture collections.

We briefly outline these below.

Salinity

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Droop (1959a) suggested that *O. marina* thrives between 4 and 130 (PSU) and indicated optimum growth at 16 PSU for the Finnish strain CCAP 1133/5. He also demonstrated an affect of acclimation on cell viability following salinity shifts: cells acclimated to 8 PSU suffered high mortality when transferred to high salinities (32-64 PSU), but cells acclimated to 64 PSU remained viable when transferred to salinities as low as 8 PSU. Lowe *et al.* (2005) demonstrated salinity tolerances between 5-55 PSU but showed that both tolerance and maximum growth varied between different strains and isolates. Lowe *et al.* (2005) also indicated two salinity responses, which appeared to correlate with habitat: intertidal strains grew best at relatively high salinities (*i.e.* >30 PSU); open-water strains grew best at low salinities (*i.e.* <30 PSU).

Temperature

Based on previously unpublished data for 10 isolates, *O. marina* can survive between 10 and 30 °C, with optimal temperatures occurring between 20 and 25 °C (Fig. 3, unpublished data). These estimates agree with data from Droop (1959a) on isolate CCAP 1133/5. He indicated an optimum temperature for growth of 22.5 °C, with higher temperatures inducing rapid decreases in growth rate and mortality ensuing at temperatures >30 °C.

pH

Only two studies have investigated the pH range that *O. marina* can tolerate (Droop, 1959a; Pedersen and Hansen, 2003). This species has a surprisingly wide pH tolerance; it can grow between pH 7.8 - 9.8. It does, however, suffer rapid mortality below pH 7.5.

Intraspecific variation

Oxyrrhis marina isolates differ genetically (Lowe *et al.* 2010) and physiologically; this includes variation in responses to temperature (Fig. 3), and salinity (Lowe *et al.*, 2005).

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Thus, the physiological ranges indicated above are likely to exceed the tolerances of many individual strains and, as a result, should be treated as guidelines.

Nutritional requirements

Oxyrrhis marina consumes an exceptionally broad range of prey species and food items covering a wide size range (Fig. 4, Supplementary Table 1). Although *O. marina* is capable of feeding on bacteria (Jeong *et al.*, 2008), the most common prey fed to *O. marina* during culturing are protists on the order of 10 to 50% its size (most commonly taxa in the genera *Isochrysis*, *Dunaliella*, and *Rhodomonas* (Fig. 4, Supplementary Table 1). For a detailed description of prey and prey preference in *O. marina*, see Roberts *et al.* (this issue).

In addition to phagotrophy, *Oxyrrhis marina* can be maintained on an entirely derived, cell-free medium. Extensive work, predominately by Droop in 1950s-70s (Droop, 1959a, b; Droop and Doyle, 1966; Droop and Pennock, 1971) established that *O. marina* could grow by osmotrophy (i.e. uptake of dissolved nutrients alone). Droop and Pennock (1971) described a fully-soluble derived medium and highlighted the unusual nutritional mode of *O. marina*. While Droop and Co-workers provided an extensive understanding of the nutritional requirements of *O. marina*, the physiological mechanisms underlying its somewhat exceptional nutritional flexibility remain unresolved. Investigating such mechanisms is likely to provide novel future research directions.

Culturing challenges for the 'omic era'

As a result of its phylogenetic position, basal to the dinoflagellates, and a suite of highly unusual cytological, genetic and genomic features, *O. marina* is increasingly the target of genetic and genomic studies aimed at understanding the evolutionary development of major protist taxa (Saldarriaga *et al.*, 2003; Cavalier-Smith and Chao 2004; Slamovits *et al.*, 2007;

1 Lowe *et al.* Isolation and culturing of *O. marina*

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3 Slamovits and Keeling 2008; Zhang *et al.* 2008). Advances in genome sequencing
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5 technologies mean that even for poorly characterised organisms, such as *O. marina*, large-
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7 scale genome and transcriptome sequencing projects can be undertaken *de novo* (Hudson
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9 2008; Vera *et al.* 2008).

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13 There is, of course, a technical issue associated with genetic and genomic studies of
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15 heterotrophic protists: heterotrophy typically necessitates using prey that also contain DNA-
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17 RNA-protein; thus, such projects begin with a ‘built-in’ contaminant. The extent of the
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19 problem is application-dependent; in small-scale genetic studies a known contaminant is
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21 simple to deal with. For example, gene- or locus- specific PCR and sequencing strategies can
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23 easily be adapted to cope with contaminants by designing organism-specific PCR primers or
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25 by size selection or cloning. However, for larger scale sequencing applications, the presence
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27 of contaminating prey organisms can be problematic. For example – “next generation”
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29 sequencing technologies allow *de novo* large-scale sequencing of organisms for which there
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31 is no prior genetic information (Hudson, 2008). In such cases, the presence of contaminating
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33 organisms in genome scale sequencing projects absorb sequence reads (increasing cost) and,
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35 more critically, can be impossible to separate from target sequence, confounding downstream
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37 analyses. Clearly then, strategies to generate ‘clean’ DNA/RNA are required to take
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39 advantage of next generation sequencing applications.
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46 We have employed three strategies for dealing with prey contamination in *O. marina*
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48 cultures to generate ‘clean’ material for mid to large scale sequencing applications. Below
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50 we summarise these and highlight some of their advantages and disadvantages for specific
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52 applications.
53

54 *Prey depletion*

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57 The simplest strategy for overcoming the presence of contaminating genetic material
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59 is to allow prey to be depleted. In our experience, in the absence of light, *O. marina* can
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graze autotrophic protists (e.g. *Dunaliella* spp., *Isochrysis galbana*) functionally to extinction. The presence, or absence, of algal cells can be determined by microscopical observations, or using PCR-based assays. For example, PCR primers designed against the 3'-end of the 18S and 5'-end of the 28S rDNA genes (to amplify the 5.8S ITS rDNA region) produces 430 and 620 bp PCR fragments for *O. marina* and *D. salina*, respectively (for primer details see Lowe *et al.*, 2005, 2010). Thus, the presence of algal cells can be determined by simple PCR and gel electrophoresis.

As an example of the above method, for a recent study developing a microsatellite library in *O. marina*, we generated 500 ml cultures at densities of $4.0\text{-}5.5 \times 10^4$ cells ml⁻¹. Cultures were established at 1×10^3 cell ml⁻¹ of *O. marina* and 5×10^5 cell ml⁻¹ *D. primolecta*. After 11 d (maintained in the dark at 16 to 20 °C), cultures appeared free of prey, but an rDNA PCR based assay was positive for *D. primolecta*. Cultures were subsequently confirmed, by PCR, to be prey-free 17 d after the initial setup. Analysis of 64 candidate microsatellite loci produced one that appeared to originate from *D. primolecta* (i.e. produced a positive result in a *D. primolecta* control PCR assay - Lowe *et al.*, submitted). This illustrates that a simple prey depletion strategy can generate 'clean' *O. marina* DNA for genome scale analyses.

Live and heat-killed Escherichia coli

An alternative to the prey depletion strategy is to use a prey organism that has a known (i.e. sequenced) and small genome. The advantage is that any DNA sequence subsequently generated can be screened against a reference genome for the prey organism and removed from further analysis. *Oxyrrhis marina* will grow on cultures of naturally occurring marine bacteria (e.g. Jeong *et al.*, 2008); though clearly, the use of an undefined (and likely complex) natural bacterial assemblage does not solve the problem of identifying contaminating sequences. Instead, the use of a well-characterised bacterium, such as *E. coli*,

1 Lowe *et al.* Isolation and culturing of *O. marina*

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3 has proved useful in our laboratory. *Oxyrrhis marina* has been demonstrated to graze *E. coli*
4 cells (e.g. Kato *et al.*, 2000), and the use of heat-killed bacteria as prey (as employed by e.g.
5 Gurijala and Alexander, 1990; Landry *et al.* 1991) means that prey numbers can easily be
6 controlled. A further advantage, for mRNA and cDNA based applications is that the
7 contribution of *E. coli* cells to subsequent RNA preparations will be minimal as bacterial
8 cells are dead and thus inactive.
9

10
11 Our protocol for feeding *O. marina* with *E. coli* is, briefly, as follows. Cultures of *E.*
12 *coli* (e.g. JM109, Promega) are grown in 50 to 500 ml Luria-Bertani broth at 37 °C in an
13 orbital incubator (210 rpm) for 8 to 12 h and culture growth monitored via optical density
14 (following standard protocols; Sezonov *et al.*, 2007). Cells are pelleted by centrifugation and
15 washed twice in sterile filtered seawater (SFSW). They are then resuspend in 5 to 50 ml
16 SFSW (~10 × concentration based on the initial volume of *E. coli* culture) and heat-killed at
17 65 °C for 30 min. Cells can be ultrasonicated, to reduce clumping and limit the occurrence of
18 bacterial flock in the final culture. The final concentration of the preparation can be
19 estimated based on optical density (we standardise our heat killed bacteria, HKB, stock
20 solutions to ~2.5 - 5.0 × 10⁸ cells ml⁻¹). A supply of 250 µl HKB in 50 ml (~1.25 - 2.5 × 10⁶
21 cells ml⁻¹) of culture every 3 to 4 d will maintain *O. marina* in exponential growth and
22 generate cell densities up to 1.5 × 10⁵ *O. marina* ml⁻¹. Treatment with antibiotics to control
23 naturally occurring bacteria in cultures is essential for use of this feeding strategy; we use a
24 penicillin and streptomycin solution (100 µg ml⁻¹) and treat with gentamycin (50 µg ml⁻¹)
25 during routine sub-culturing at 1-2 week intervals.
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28 *A simplified axenic medium*

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30 Research by Droop and co-workers (Droop, 1959a,b; Droop and Doyle, 1966; Droop
31 and Pennock, 1971) indicated that *O. marina* can be grown indefinitely on a medium
32 containing solely water soluble factors, highlighting that *O. marina* does not rely exclusively
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on phagotrophy for survival. Clearly, in a research context, the ability to culture *O. marina* cells axenically is potentially useful in generating material for genetic analyses. In our laboratory, Droop's axenic medium supports growth of a range of genetically diverse *O. marina* isolates, suggesting that most 'variants' of *O. marina* can grown axenically on a soluble nutrient diet.

The commercial availability of readymade culture media and nutritional supplements has allowed us to simplify Droop's original axenic medium (see Table 1). Our experiments with Droop's Finnish strain of *O. marina* (CCAP 1133/5) and two other strains (44_PLY01, Lowe *et al.*, 2010; and CCMP 1788) generated cell densities up to 1.5×10^5 cells ml⁻¹ in axenic culture using our modified recipe (Table 1). Microscopical observations showed that the cells were morphologically similar to those grown on a phytoplankton diet. In addition, the axenic cells contained similar quantities of lipid and membrane protein (200 µg membrane protein, 10⁷ cells⁻¹) as their phytoplankton raised counterparts (Wootton, pers. obs). However, one notable discrepancy occurred: strain CCMP 1788 lost its pink pigmentation during axenic culture and the cells became opaque and white. Despite this, the cells remained active and otherwise morphologically indistinguishable from other cultures (Wootton pers. obs). We have not pursued the causes of pigmentation loss in this strain under axenic culturing, though clearly the occurrence of differences in strain responses is notable in context with other observations of genetic and physiological diversity within *O. marina* (Lowe *et al.*, 2005, 2010).

The success of growing visibly 'healthy' *O. marina* cells under axenic conditions increases the potential utility of this protist as a valuable model organism, highlighting the organism's robustness. Further, this procedure overcomes problems associated with DNA-RNA-protein contamination inherent in the genetic and genomic study of heterotrophic protists.

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SUMMARY

Free-living protists represent a relatively understudied component of total microbial diversity (Caron *et al.* 2009); as a result detailed genetic, physiological and behavioural study of an increasingly broad range of model protist taxa represents an important focus of contemporary microbiological research. This review emphasises why *O. marina* has proved a useful model protist: it is abundant, relatively simple to isolate from the environment and straightforward to maintain in the laboratory. Perhaps more importantly, this practicality means that *O. marina* is likely to be a useful target for the study of a large range of questions in an ecological, physiological and evolutionary context. For example, the relatively large numbers of *O. marina* strains that can be practically isolated (10^2 - 10^3) lends this organism to the study of population level processes and parameters, such as dispersal and gene flow, effective population size and adaptation – parameters and processes that are poorly characterised for free-living protists in general (Martiny *et al.* 2006; Caron *et al.* 2009).

It is notable from nutritional and ecophysiological studies that *O. marina* is incredibly flexible in its feeding requirements - able to consume a broad range of prey items but also to feed by osmotrophy. Thus, while the process of culturing is most often a means to an end, it is clear that for *O. marina*, explicit culturing studies to examine the physiological and biochemical basis of feeding mechanisms and nutrition are likely to be interesting avenues for future research (such directions are examined in more detail by Roberts *et al.*, this issue). *Oxyrrhis marina* also displays broad environmental tolerances; in particular, it is eurytopic with regard to salinity, temperature and pH. However, there is some evidence for ecophysiological differences between strains; thus, the study of large numbers of isolates may prove useful to assess functional diversity and processes of environmental adaptation.

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Finally, in light of the increasing interest in the study of the genetics, genomics and proteomics of *O. marina* we highlight three strategies employed in our laboratories for generating DNA/RNA/protein suitable for such applications. In particular we highlight the use of heat killed *E. coli* as a useful, robust, and above all, simple strategy for generating *O. marina* cultures free of other eukaryotic organisms. Beyond the technical challenges associated with culturing, however, the task of developing genomic resources for *O. marina* still remains; studies describing novel chromosome structure (Triemer, 1982; Gao and Li, 1986), unusual RNA editing mechanisms (Slamovits *et al.*, 2007; Zhang and Lin, 2008), and potentially enormous genome size (Sano and Kato, 2009), suggest that the development of such resources is likely to be a complex and time consuming process.

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The authors would like to thank Zhou Yang for providing the temperature vs growth rate responses for *O. marina* isolates (Fig. 3). Michael Steinke provided constructive comments on the manuscript and a large number of friends, family and colleagues, too numerous to mention, assisted with sample collection. We would also like to thank two anonymous reviewers for their comments on a previous version of the manuscript.

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FIGURE LEGENDS

Fig. 1. A summary of sampling and culturing effort in the microzooplankton laboratory (University of Liverpool) between April 2008 and September 2009. **a)** *Oxyrrhis marina* was recovered from ~50% of water samples, collected predominantly from intertidal habitats; ~90% of these samples were successfully established as cultures. **b)** The number of samples collected for each one month period (April 2008 - September 2009). Filled bars indicate numbers of water samples collected; open bars indicate the number of samples that contained *O. marina*. These sampling data indicate that *O. marina* can be collected from intertidal habitats throughout the year. **c)** Effect of sampling effort (number of samples collected from a location) on the probability of recovering *O. marina* from an intertidal environment (i.e. the likelihood of obtaining at least one water-sample that contains *O. marina*). The probability of recovering *O. marina* increases from 0.4 when a single samples is collected to ~1.0 when >15 samples are obtained. **d)** Establishment rate of single cells isolated from environmental samples. For each sample, cells were isolated into 24 well (2 ml tissue culture plates) – on average 40% of isolates result in established cultures.

Fig. 2. Light micrographs of *Oxyrrhis marina*. **a)** and **b)** acidified Lugol's iodine fixed (2%) environmental samples from littoral tide pools, Port St. Mary, Isle of Mann; *O. marina* cells are a characteristic 'rugby ball' shape with a posterior 'notch'. In high-shore tide pools (*i.e.* above mean high water neap tide), *O. marina* may reach densities on the order of 10^3 cells ml^{-1} . **c)** *Oxyrrhis marina* cells in monoclonal culture undergoing division. **d)** Vegetative *O. marina* cells; visible are the tentacular lobe (h), and the longitudinal (l) and transverse (t) flagella (see Lowe *et al.*, this issue, for a description of gross morphology)

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Fig. 3. Combined growth rate responses of nine *Oxyrrhis marina* isolates to temperatures between 15 and 30 °C. Growth rate responses to temperature are highly variable between strains (unpublished data, Z. Yang in prep).

Fig. 4. A summary of prey species/food sources demonstrated to allow growth of *Oxyrrhis marina* in laboratory cultures. Data are synthesised from 72 peer-reviewed manuscripts published between 1953 and 2008.

For Peer Review

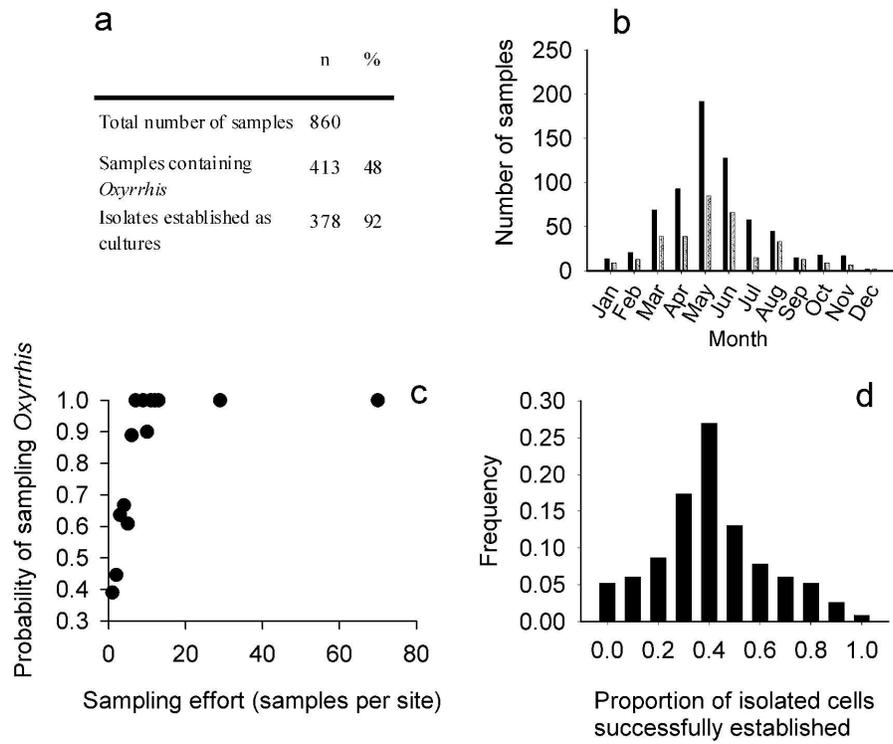
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Table. 1. Components of a medium for the axenic culturing of *Oxyrrhis marina*. The medium is a modification of that presented by Droop (1959b).

Component	Stock solutions	Final (~1L) medium
GF/C filtered autoclaved seawater (32 PSU)	-	750 ml
Amino acid/Sodium acetate solution (pH 7.5), containing: <ul style="list-style-type: none"> • Glycylglycine hydrochloride (<i>e.g.</i> Sigma G1127) • L- valine (<i>e.g.</i> Sigma 94619) • L- proline (<i>e.g.</i> Sigma P5607) • Sodium acetate 	300 ml 5 g 2.5 g 0.4 g 20 g	22.5 ml
F/2 medium without silicate (<i>e.g.</i> Sigma G0154)	-	20 ml
Cholesterol solution (8mg/ml), containing: <ul style="list-style-type: none"> • water soluble cholesterol (<i>e.g.</i> Sigma C4951) 	5 ml 40 mg	5 ml
Coenzyme Q ₁₀ solution (100 µg/ml), containing: <ul style="list-style-type: none"> • 2 mg coenzyme Q₁₀ in 1 mL ethyl acetate added to 19 ml 0.2% glycerol trioleate in absolute ethanol* 	20 ml	200 µl
1× Leibovitz L-15 medium (<i>e.g.</i> Gibco 21083) + 2.4% (w/v) NaCl	-	200 ml

* preparation of Coenzyme Q₁₀ follows protocol of Droop and Pennock (1971)

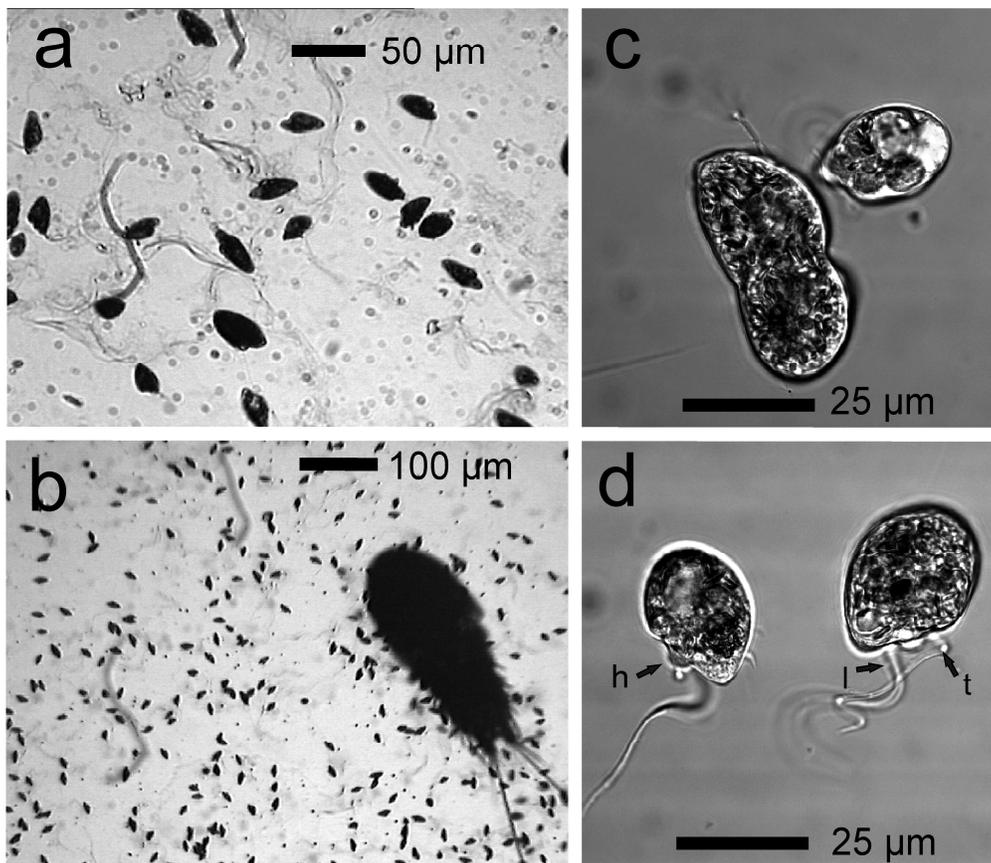
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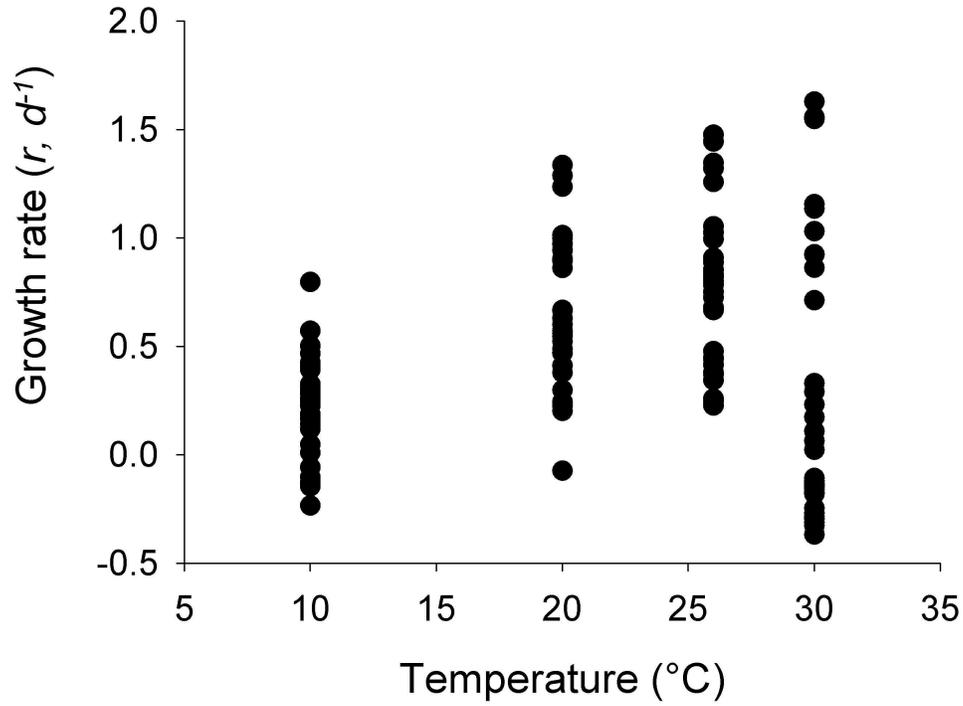
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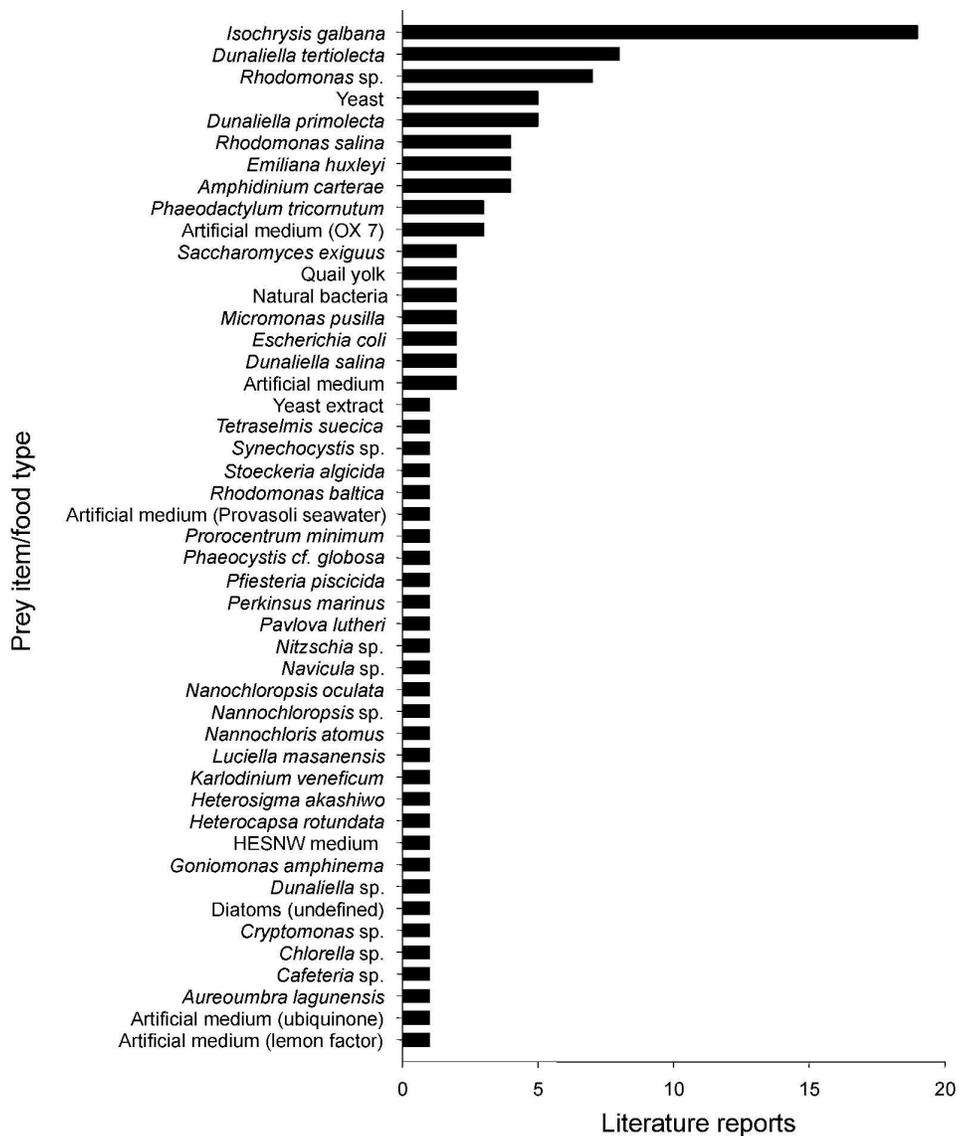
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Supplementary Table. 1. A summary of prey species/food sources demonstrated to allow growth of *Oxyrrhis marina* in laboratory cultures, including supporting literature citations. Data are synthesised from 72 peer-reviewed manuscripts published between 1953 and 2008.

Prey item/food type	Literature reports	Reporting Articles
<i>Amphidinium carterae</i>	4	Jeong <i>et al.</i> (2001), Jeong <i>et al.</i> (2004), Jeong <i>et al.</i> (2007), Jeong <i>et al.</i> (2003)
Artificial medium	2	Droop (1953), Gao and Li (1986)
Artificial medium (lemon factor)	1	Droop (1959a)
Artificial medium (OX 7)	3	Droop and Pennock (1971), Slamovits and Keeling (2008), Slamovits <i>et al.</i> (2007)
Artificial medium (ubiquinone)	1	Droop and Doyle (1966)
Artificial medium (Provasoli seawater)	1	Hohfield <i>et al.</i> (1994)
<i>Aureoumbra lagunensis</i>	1	Buskey <i>et al.</i> (1998)
<i>Cafeteria</i> sp.	1	Jeong <i>et al.</i> (2007a)
<i>Chlorella</i> sp.	1	Hammer <i>et al.</i> (1999)
<i>Cryptomonas</i> sp.	1	Strom <i>et al.</i> (1997)
Diatoms (undefined)	1	Roberts (1985)
<i>Dunaliella primolecta</i>	5	Flynn and Fielder (1989), Flynn <i>et al.</i> (1996), Martel and Flynn (2008), Opik and Flynn (1989), Roberts <i>et al.</i> (2006)
<i>Dunaliella salina</i>	2	Lowe <i>et al.</i> (2005), Tang and Taal (2005)
<i>Dunaliella</i> sp.	1	Klein Breteler <i>et al.</i> (1999)
<i>Dunaliella tertiolecta</i>	8	Buskey <i>et al.</i> (1998), Carotenuto <i>et al.</i> (2002), Chu <i>et al.</i> (2008), Goldman <i>et al.</i> (1989), Hartz <i>et al.</i> (2008), Lund <i>et al.</i> (2008), Lund <i>et al.</i> (2008), Roberts (1993)
<i>Emiliana huxleyi</i>	4	Evans <i>et al.</i> (2007), Hansen <i>et al.</i> (1996), Strom <i>et al.</i> (2003), Strom <i>et al.</i> (2003a)

1		2	Kato <i>et al.</i> (1997), Kato <i>et al.</i> (2000)
2	<i>Escherichia coli</i>		
3		1	Martin-Cereceda <i>et al.</i> (2008)
4	<i>Goniomonas amphinema</i>		
5	HESNW medium	1	Saldarriaga <i>et al.</i> (2003)
6			
7	<i>Heterocapsa rotundata</i>	1	Jakobsen and Strom (2004)
8			
9	<i>Heterosigma akashiwo</i>	1	Jeong <i>et al.</i> (2003)
10			
11	<i>Isochrysis galbana</i>	19	Archer <i>et al.</i> (1996), Archer <i>et al.</i> (2001), Barlow <i>et al.</i> (1988), Buskey <i>et al.</i> (1998), Davidson <i>et al.</i> (1995), Flynn and Davidson (1993), Flynn and Davidson (1993a), Flynn <i>et al.</i> (1996), Goldman <i>et al.</i> (1989), Hansen <i>et al.</i> (1996), Hansen <i>et al.</i> (1993), Kimmance <i>et al.</i> (2006), Martel (2006), Martel and Flynn (2008), Menden-Deuer and Grunbaum (2006), Mitra and Flynn (2005), Oosterhuis <i>et al.</i> (2000), Pelegri <i>et al.</i> (1999), Tang and Taal (2005)
12			
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14			
15	<i>Karlodinium veneficum</i>	1	Adolf <i>et al.</i> (2007)
16			
17	<i>Luciella masanensis</i>	1	Jeong <i>et al.</i> (2007)
18			
19	<i>Micromonas pusilla</i>	2	Flynn <i>et al.</i> (1996), Hansen <i>et al.</i> (1996)
20			
21	<i>Nannochloris atomus</i>	1	Davidson <i>et al.</i> (2005)
22			
23	<i>Nannochloropsis sp.</i>	1	Hansen <i>et al.</i> (1996)
24	<i>Nanochloropsis oculata</i>	1	Pond <i>et al.</i> (2006)
25			
26	Natural bacteria	2	Hammer <i>et al.</i> (1999), Stevens <i>et al.</i> (2004)
27			
28	<i>Navicula sp.</i>	1	Clarke and Pennick (1976)
29			
30	<i>Nitzschia sp.</i>	1	Clarke and Pennick (1976)
31			
32	<i>Pavlova lutheri</i>	1	Davidson <i>et al.</i> (2005)
33			
34	<i>Perkinsus marinus</i>	1	Lund <i>et al.</i> (2008)
35			
36	<i>Pfiesteria piscicida</i>	1	Jeong <i>et al.</i> (2007)
37			
38	<i>Phaeocystis cf. globosa</i>	1	Hansen <i>et al.</i> (1993)
39			
40	<i>Phaeodactylum tricornutum</i>	3	Archer <i>et al.</i> (1996), Barlow <i>et al.</i> (1988), Goldman <i>et al.</i> (1989)
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42	<i>Prorocentrum minimum</i>	1	Jeong <i>et al.</i> (2001)
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1		2	Kato <i>et al.</i> (2000), Osaka <i>et al.</i> (2003)
2	Quail yolk		
3		1	Mariani <i>et al.</i> (2008)
4	<i>Rhodomonas baltica</i>		
5		4	Carvalho and Granéli (2006), Chu <i>et al.</i> (2008), Pedersen and Hansen (2003), Tang and Taal (2005)
6	<i>Rhodomonas salina</i>		
7		7	Bartemeus <i>et al.</i> (2003), Grossi <i>et al.</i> (1996), Hansen <i>et al.</i> (1996), Jakobsen and Strom (2004), Klein Breteler <i>et al.</i> (1999), Klein <i>et al.</i> (1986), Oosterhuis <i>et al.</i> (2000)
8	<i>Rhodomonas</i> sp.		
9		2	Droop (1953), Droop (1959)
10	<i>Saccharomyces exiguus</i>		
11		1	Jeong <i>et al.</i> (2007)
12	<i>Stoeckeria algicida</i>		
13		1	Hammer <i>et al.</i> (1999)
14	<i>Synechocystis</i> sp.		
15		1	Hansen <i>et al.</i> (1996)
16	<i>Tetraselmis suecica</i>		
17	Yeast	5	Cachon <i>et al.</i> (1988), Cosson <i>et al.</i> (1988), Cosson <i>et al.</i> (1996), Gognon <i>et al.</i> (1994), Godart and Hoitorel (1992)
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19	Yeast extract	1	Brown <i>et al.</i> (1988)

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