Chapter 7: Molecular diagnostic solutions in algal cultivation systems

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Abstract:
Contamination of algal mass culture by predators, pathogens and competitors represent one of the major challenges to commercial production of algae. Early detection of, and response to, these deleterious organisms dictates how much of an effect they will have on the algae biomass. Thus, the incorporation of rapid detection tools into an algae production routine is crucial. Molecular methods of identification and detection represent alternative, high throughput tools that can supplement, and in some cases replace, optical methods for the identification and detection of deleterious species in algal mass culture systems. Many of these molecular methods are quite new and their use has been increasingly demonstrated across disciplines in recent years. Here, we review these techniques, including Next Generation Sequencing, qPCR, microarrays, and Fluorescent *in situ* hybridization (FISH), and comment on their promise and limitations. Finally, we discuss how some of these modern methods have been used to detect biocontaminants in algae production systems and the various responses these type of data can direct.

7.1. Introduction

One of the major challenges to achieving high rates of long-term production in microalgal mass cultures is the elimination or reduction of the impact of contamination and crashes in production systems. Although there are both biotic and abiotic root causes of mass culture crashes, infection by deleterious species may be one of the least understood. Aside from a few well-known culprits, the diversity of pathogens, parasites, predators and weed species has not been well characterized. Lost production days due to pond crashes can significantly lower annualized production rates. Depending on the scale and type of system, several weeks of production can be lost while the system is disinfected and new inoculum and growth medium is prepared. Depending on the design and operation of the production facility, there is a risk of spread or persistence of contamination and successive crashes. Despite a paucity of publically available data on the economic impact of biocontaminants on the nascent algae biomass industry, the consensus is that they constitute an economic barrier to commercialization (ANL, NREL, PNNL 2012, Gao et al. 2012). Some insight into the potential magnitude of the financial impact may be gained from the aquaculture-for-food industry which loses several billion US$ annually (Subasinghe et al. 2001, FAO 2010) due to bacterial and fungal infections (Defoirdt et al. 2004, Ding and Ma 2005, Ramaiah 2006).

Although some organisms such as certain bacteria and protozoa may stimulate algal productivity (Cole 1982; Kazamia et al. 2012), there are a wide variety of deleterious species. These include bacteria (Cole 1982, Fukami et al. 1997), viruses (Dunigan et al. 2006), parasites (e.g., Letcher et

al. 2013), fungi (Fott 1967, Becker, 1994, Hoffman et al. 2008, Li et al. 2010) and herbivorous zooplankton (e.g., Park et al. 2011). In addition there are algal "weed species" (Pienkos and Darzins 2009,Bull and Collins 2012) that can reduce the value of the biomass by supplanting the desired species. Early detection of deleterious species is the key to informed pond management strategies and thus successful large-scale algae cultivation will require routine, detailed, fast and cost-effective identification of potentially deleterious species that have become established in the culture and how their populations may change with time and conditions.

7.2. Diagnostic methods

Identification of algal pests is still largely dependent on microscopy (e.g., Rasconi et al. 2009). Although optical methods can produce near real time data on the presence of contaminants, microscopy is a method that is labor intensive, low throughput and requires a certain level of expert knowledge to recognize contaminant species. As a consequence of these limitations a variety of advanced optical methods have recently been developed such as flow cytometry coupled with digital imaging and image recognition (reviewed by Álvarez et al. 2011, Day et al. 2012). While such methods represent potential improvement over standard optical methods these new methods are still dependent on *a priori* knowledge of deleterious species. In addition, these techniques can severely underestimate diversity of some groups (Fenchel et al. 1997).

Molecular diagnosis, in general, can be divided into two separate processes. The first is the initial identification of novel etiological agents: viruses, predators, pathogens and parasites. Once identified and characterized, assays can be developed against these agents that enable the second process namely, the detection of known agents in algal cultures. Initial identification of novel agents is still, fundamentally, based on the postulates first formulated by Robert Koch in the late nineteenth century. Classical methods of fulfilling Koch's postulates have depended upon the isolation and culturing of the etiological agent and the demonstration that the disease state is unambiguously correlated with the presence of the pathogen via re-isolation. However. in the last two decades culture-free molecular methods of identification and detection have become more commonplace in clinical diagnostics and there are a number of instances in which the etiological agent is not culturable and is recognized by its molecular signature only. Most recently, low cost methods of next generation sequencing have revolutionized pathogen discovery. The rise of molecular diagnostics in general has lead to a rethinking of Koch's postulates (Fredericks and Relman 1996). It is now reasonable to reformulate Koch's postulates for molecular diagnosis of algal production system infections:

1. A nucleic acid sequence belonging to the putative pathogen, predator, or parasite should be present in an infected algal mass culture.

2. Fewer, or no, copies of pathogen-associated nucleic acid sequences should occur in healthy mass cultures.

3. When sequence detection predates mass culture infection, increase in sequence copy number should correlate with loss of algal biomass, decrease in productivity or aberrant pond performance.

4. The nature of the deleterious organism, inferred from the available sequence, should be consistent with the known biological characteristics of that group of organisms.

Several factors can confound the identification of novel pest species by molecular analysis of algal mass culture infections. Other sequences may become prevalent in crashed or infected ponds including those related to species that feed upon detritus or dead algal cells. In these cases, temporal relationships are critical to establish and thus the frequency of pond sampling is an important consideration. A reasonable strategy is to collect and archive samples on frequent and routine basis to enable the analysis of a time-course of infection should a deleterious event occur. Environmental conditions and abiotic stressors may play a significant role in mediating pond crashes and can be in themselves the root cause of pond crashes. Poly microbial infections are possible although many crashes appear to be caused by a single agent (Hu pers comm).

7.2.1 *Deleterious species identification by next generation sequencing.*

Next Generation Sequencing (NGS) has been employed extensively to identify unknown pathogens in a diversity of situations ranging from humans, to vineyards, to beehives. NGS based approaches have recently been applied to the characterization of the predator, pathogen and parasite loads of underperforming and crashed pilot scale ponds and photobioreactors (Carney et al. unpublished data, Carney et al. 2013). The sequencing strategies employed in such analyses are dependent on the read length and number which are in turn dependent on the technical specifications of the sequencing system employed. There are a number of sequencing systems in use today and the most common are summarized in Table 1.

Table 1 current next generation sequencer capabilities

Sequencer	Read lengths	Read number	Run time
Illumina Hi Seq	100 nt	6 $X10^9$	2-11 days
Illumina MiSeq	600 nt	1.2-1.5 $X10^7$	39 hrs
454 GS	400 nt	7 $X10^4$	10hrs
GS FLX	700 nt	1 $X10^6$	23 hrs
Ion Torrent PGM	Up to 400 nt	Up to 6 $X10^6$	2-7 hours
Ion Torrent PI	Up to 200 nt	Up to 3.3 $X10^8$	2-4 hours
Pacific Biosciences	4300-5000 nt	2.5-5.0 $X10^3$	~2 hours
Applied biosystems Solid 5500	50-100 nt	1 $X10^8$	1-7 days

Generally sequencers fall into two broad categories, high volume machines that are found in core facilities and commercial providers and less expensive, lower volume "personal" sequencers. Personal sequencers including the Illumina MiSeq, 454 GS, and the Ion Torrent PGM have the advantage of relatively rapid run times and lower cost per run than the larger machines. Pacific Biosciences sequencers are targeted for specific applications requiring a relatively small number of long reads. The costs associated with the actual sequencing can be reduced by the creation of multiplexed sequencing libraries allowing for the sequencing of more than one sample per lane. Various systems that take advantage of each type of sequencer have been developed for the

creation of multiplexed libraries (Mckenna et al. 2008, Caporaso et al. 2011, Whiteley et al. 2012).

There are several regions that have been employed for microbiome analysis of clinical and environmental samples (Hoef-Emden 2012) including the large and small subunit ribosomal RNA genes, the internal transcribed spacer of the ribosomal RNA genes and the mitochondrial cytochrome oxidase gene. The best known of these targets is a small subunit ribosomal RNA gene (SSU rRNA). The prokaryotic SSU rRNA gene has nine hypervariable regions dispersed along its length (Van De Peer et al. 1996). Of these, the sequence information contained in the individual hypervariable regions three or six (Huse et al. 2008) or a fragment covering hypervariable regions 1 through 3 or 4 (Kim et al. 2011) are the most useful for phylogenetic determination. Sequence data from individual hypervariable regions is often sufficient for genus level distinction. Where as data from multiple hypervariable regions or the entire SSU rRNA gene can result in species level distinction.

The eukarytotic SSU rRNA gene also contains nine variable regions of which V4 and V9 are the most informative and are often used in combination (Amaral-Zettler et al. 2009, Pawlowski et al. 2011, Stoeck et al. 2010, Orsi et al. 2013). The V4 region is the longest with the highest degree of length variation and sequence heterogeneity (Nickrent and Sargent 1991). The information contained in either region is generally sufficient for the genus level identification of an organism. The shorter V9 region is less useful partially because it is sometimes not present in truncated versions of eukaryotic SSU rRNA genes found in sequence databases. Although phylogenetic analysis based on SSU rRNA gene sequence is more common, similar analysis based on the large subunit rRNA (LSU rRNA) is also carried out (Ludwig and Schleifer 1994). The LSU rRNA gene is longer and contains two variable regions, D1 and D2, that can be used for phylogenetic analysis (Sonnenberg et al. 2007, Putignani 2008)

In samples that contain high algae biomass densities, prokaryotic rRNA gene sequencing libraries may be dominated by amplicons derived from the algal chloroplast. In these cases, primers devised to exclude amplification of plastid SSU rRNA sequences can be used in single step or in nested PCR amplifications (Rastogi et al. 2010, Chelius and Triplett 2001). It should be noted that these primers also exclude the amplification of cyanobacterial sequences. Other strategies for reducing the burden of non-informative sequences include the use of so-called blocking primers that bind to and prevent amplification of unwanted sequences. Such primers contain modified nucleotides that prevent primer extension (Vestheim and Jarman 2008). In theory blocking primer strategies may be more generalizable and could be applied to additional amplification targets aside from rRNA genes.

The intergenic transcribed spacer (ITS) region, located between the rRNA genes encoding the SSU and the LSU, is commonly used for genus or species level discrimination in fungal phylogenetics and there is a very large database of fungal ITS sequences (REF). In eukaryotes the ITS region (See figure 1C) consists of two hypervariable spacers, ITS1and ITS2, that flank the gene encoding the 5.8s ribosomal subunit. A full length amplicon including both ITS1 snd ITS2 regions and the 5.8s subunit is approximately 650 bp in length which is beyond the current read length limits of many of the next generation sequencers (see Table 1). Consequently, Individual ITS regions have been analyzed by next generation sequencing (Lindner et al. 2013).

Reports indicate that community analyses based on ITS1 versus ITS2 yield different taxonomic compositions from each other as well as from those based on the full length ITS region.

The 5' terminus of the mitochondrial cytochrome oxidase gene *cox1* has been used as a molecular barcode region primarily for metazoans (Bucklin et al. 2011). There are caveats to the use of *cox1*: the amplification of nuclear encoded pseudogenes can lead to overestimation of species diversity (Song et al. 2008) and it has proven difficult to develop universal primers (Saunders and McDevit 2012).

The advantages of next generation sequencing based methods of identification are that they are culture independent and do not rely on isolation of the organism. If however, the deleterious organism or weed species is of sufficient abundance in the contaminated culture or has been isolated or enriched in culture, it is possible to clone and carry out dideoxynucleotide terminator sequencing (Sanger et al. 1977) of the desired region. With so-called Sanger sequencing it is generally possible to achieve 700 nt of sequence data per primer extension reaction. Thus, paired end reactions should be sufficient to sequence both DNA strands of the ITS region and single strands of most if not all of the full length SSU rRNA genes. By providing full sequencing coverage of the region of interest, Sanger sequencing can result in a high degree of taxonomic distinction of the target organism and maximal information for the design of PCR primer or oligonucleotide probes for the future detection and quantification of the deleterious species in algal mass culture.

Methods for phylogenetic analysis that originally were developed using the relatively long read lengths generated by Sanger sequencing are being adapted for microbiome analysis by next generation sequencing. These methods have then evolved as the read lengths have increased dramatically for each type of sequencer. For example, when introduced, Illumina reads were limited to 36 nucleotides (nt). Now it is possible, with the Illumina MiSeq, to obtain reads that span regions of up to 600 nt using paired end kits. Taking prokaryotic SSUrRNA gene analysis as an example, the MiSeq is now capable of completely sequencing amplicons covering hypervariable regions 1-3. An alternative strategy for obtaining full length rRNA sequence coverage, with next generation sequencers, entails amplifying the full length gene then shotgun sequencing and assembling the amplicon. This strategy is more complex because it requires an assembly step that is not necessary for the analysis of shorter amplicons. The advantage is that more information is available upon which to base phylogenetic assignments.

Algal viruses may be the greatest source of un-characterized genetic diversity in the world (Dunigan et al. 2006). Those that have been able to be cultured include 40 representatives, infecting 11 microalgae species, and have all been characterized as large dsDNA viruses (family *Phycodnaviridae*; Nagasaki and Bratbak 2010). Although, to date, pond crashes due to viral infection have not been reported, it is clear that algal viruses play a significant role in the collapse of algal blooms in natural ecosystems and it is inevitable that viral pathogens will impact algal mass culture. Although PCR amplification of specific sequences related to the phycodnaviridae has been demonstrated in marine samples (e.g., Chen and Suttle 1995), there have been no universal molecular barcode regions developed for viruses in general. Thus, the identification of novel viruses is dependent on shotgun sequencing of purified viral nucleic acid fractions followed by genomic assembly. Methods have been developed in both freshwater and

marine systems to concentrate viral particles and prepare the nucleic acids for sequencing (Lawrence and Steward 2010).

Key concerns, when utilizing molecular diagnostic methods such as sequencing or qPCR, are lysis and extraction of nucleic acids from the biomass sample. Differential or failure of extraction of nucleic acids from one group of organisms can skew apparent relative abundances. A variety of protocols and commercial kits for the extraction of nucleic acids from recalcitrant organisms have been developed and there are some comparisons of these methods reported in the literature (Purdy 2005, Koid et al. 2012). However, new methods and commercial kits are routinely developed and it is advisable to test the effectiveness of a variety of protocols on the sample types of interest using the intended molecular diagnostic method (qPCR, sequencing, etc.). Many lysis and extraction protocols feature a combination of both chemical and mechanical lysis methods. There are several forms of mechanical lysis including sonication, French pressure cell, nitrogen bomb, cyropulverization and bead beater. For field applications bead beating is arguably the most convenient form of mechanical lysis. This is largely due to the potential low cost of instrumentation and ability to handle small sample volumes. For particularly recalcitrant samples, an alternative method is cryopulverization in which the sample is first flash frozen in liquid nitrogen then pulverized with a cold mortar and pestle.

In addition to bias introduced in the nucleic acid extraction, additional bias in molecular diagnosis techniques can be introduced by PCR (both for detection and sequencing library preparation) and potentially, by the choice of sequencer. There are two major sources of PCR bias, the choice of primers for amplification and the reaction conditions used for amplification. Specific primers for detection are designed for either universal or taxa specific amplification. It has, however, proven difficult to create primer sets that amplify all targeted sequences with equal efficiency. This bias can be characterized and potentially limited by choice and testing of a variety of primers against a diversity of targets species or near neighbors. It may be desirable to compare libraries created with alternative primer sets targeting either the same region or different molecular barcodes. PCR amplification bias at high and low GC ratios is also a potentially significant source of error introduced during the generation of sequencing libraries (Aird et al. 2011). This can be controlled or eliminated by adjustment of the PCR cycle parameters and choice of polymerase. In addition to other sources of bias, each next generation sequencing systems displays a different level of bias against templates with at high and low GC ratios (Quail et al 2012).

7.2.2 *Bioinformatics for molecular diagnosis*

The handling of the raw sequence data from the sequencer is device-dependent and instruments generally come with data process and analysis software packages. Raw sequencing reads must pass through a series of quality control steps to remove low quality and primer sequences and to mask low complexity regions. Once this initial processing is complete there are a number of software packages for microbiome analysis including QIIME http://qiime.org/index.html. (Caporaso et al. 2010), Mothur (Schloss et al 2009), and the RDP pyrosequencing pipeline designed for the analysis of 454 sequencing datasets.

There are a number of sequence data repositories for rRNA and mitochondrial cytochrome oxidase genes. The SILVA database, http://www.arb-silva.de/, is a curated repository of SSU and LSU rRNA gene sequences from archaea, bacteria and eukarya (Quast et al 2013). The Greenenes database contains LSU rRNA sequences from bacteria and archaea http://greengenes.secondgenome.com/downloads. Both databases can be downloaded from their websites in a flat file format for use with the various microbiome analysis software packages. The RDP Ribosomal Database project, http://rdp.cme.msu.edu/, (Cole et al. 2009) maintains a database of bacterial and archaeal SSU sequences. UNITE (Abarenkov et al. 2010) is a searchable data base of primarily fungal ITS regions which can be downloaded in flat file format from either from its home website, http://unite.ut.ee/, or from the QIIME website. As the name suggests, ITS2 database (Koetschan et al 2012) is a searchable database of specifically ITS2 sequences, http://its2.bioapps.biozentrum.uni-wuerzburg.de/ and ITSoneDB, http://itsonedb.ba.itb.cnr.it/ focuses on the ITS1 region. The BOLD database (Ratnasingham and Hebert 2007), www.barcodinglife.org, contains both sequence data and a registry of primers for *cox1*. Genbank is the NIH depository of publically available annotated sequences, (Benson et al. 2012) http://www.ncbi.nlm.nih.gov/genbank/ and, as a last resort, can be queried to identify sequences that are not found in other databases.

Once the deleterious agent or weed species of concern has been identified and sufficient sequence information is available, bioinformatics analysis is employed in the development of oligonucleotide primers and probes for the detection and quantification of the agent in algal mass culture. The rRNA gene databases SILVA, Greengenes, and RDP host web based tools to assist in the development of probes by checking them for homology to sequences in their respective databases. The web tool probeCheck, http://131.130.66.200/cgi-bin/probecheck/content.pl?id=home (Loy et al. 2008) provides a single interface for the comparison of rRNA subunit targeted probe sequences against multiple databases.

7.2.3. *Quantitative PCR*

Quantitative PCR (qPCR) is one of the more common molecular diagnostic techniques for known waterborne agents (for review see Botes et al. 2013). This method has several advantages the most important of which is the potential for high sensitivity. Under the appropriate conditions, qPCR can approach single cell detection levels. In practice this level of sensitivity can be difficult to obtain and care must be taken in the preparation of samples to avoid contamination. qPCR reactions can be multiplexed for the detection of multiple species within a single reaction and given the appropriate equipment, thousands of such reactions could be run on a daily basis. The limitations to qPCR, as a detection technology, include the initial costs of the equipment. The care must also be taken in the development and validation of specific primers.

Two different reporter systems for qPCR are in general use; fluorogenic dyes or fluorescent oligonucleotide probe. The first system (Ponchel *et al* 2003) utilizes dyes, such as Sybr Green, that fluoresce when bound to the double stranded product of PCR reactions allowing quantification of the product. The limitation of this system is that the dye binds non-specifically to any double stranded DNA including primer dimers. The major advantage of the fluorgenic dye based system is that it is less expensive than probe based system. Probe based systems

(often referred to as Taqman) utilize a fluorescently labeled oligonucleotide that binds to the desired product (Holland *et al* 1991). This allows for greater specificity and enables quantification of the target even in the presence of non-target amplicons. In addition, probe based systems can be multiplexed for detection of multiple species. The disadvantage of such systems is that the reactions are more expensive because of the requirement for the labeled oligonucleotide probe.

7.2.4. *Microarray based methods*

Microarray based systems have been developed using arrayed oligonucleotide probes targeted to the SSUrRNA. These so-called phylochips were originally developed for prokaryotic community structure analysis and there is an extensive literature on their use (Lot et al. 2002). Custom phylopchips can be developed for the detection of organisms of interest and the phylochip method was adapted to eukaryotic SSU rRNA analysis and applied to the detection of marine eukaryotes, including microalgae and pathogenic protozoa (Metfies et al. 2007). A potential drawback to Phylochip based pond diagnostics is the expense of microarray development and production, particularly in situations when there are few species to be monitored. Macroarray systems, based on dot blot technology can be utilized for rapid optimization and validation of probes or for applications where only a modest number of species are to be detected.

7.2.5. *Flurorescent in situ hybridization*

Fluorescent *in situ* hybridization (FISH) has been applied to the identification and enumeration of morphologically indistinguishable species such as bacteria and archaea (DeLong, et al 1999). The method entails hybridization of permeabilized samples with fluorescently labeled olignucleotide probes followed by fluorescent imaging or flow cytometry. The method is technically challenging, expensive and time consuming and is unlikely to be applied to routine mass culture diagnostics or surveillance.

7.3. Identifying problematic contamination

Molecular techniques are very useful in characterizing the community within an algae cultivation system. These techniques are used to not only verify the intended strain but also to detect biocontaminants before they become an irreversible problem. In cases where a natural community of algal strains is allowed to colonize a cultivation system, molecular tools can also be quite valuable. Below we describe some potentially deleterious biocontaminants and give examples where molecular techniques were used to detect them.

7.3.1 *Mesograzers*

Growing algae to scale will undoubtedly lead to some infestations by algivorous predators. Metazoan rotifers are perhaps the most commonly reported biocontaminant in large scale cultivation systems. Once established, rotifers cause extremely rapid biomass loss as they are able to double their density in 24 hrs (Yúfera and Navarro 1995, Sarma et al. 2001) while ingesting 200 algal cells min^{-1} $rotifer^{-1}$ (Hirayama and Ogawa 1972). Rotifers also form resting

stages that are resistant to desiccation and may also be resistant to treatment with chlorine. However, other lesser known grazers are also problematic, including amoebae, heliozoans, and vampyrellids, and further characterization of large scale cultures will be needed to further investigate the specific threat they pose to commercial algae productivity.

Replicate raceway ponds in Texas growing *N. salina* were reported to simultaneously crash, presumably from different organisms. Microbiome analysis using NGS confirmed that one crash was due to the outbreak of the rotifer, *Brachionus plicatilis*, while the other was attributed to a mix of the grastrotrich, *Cheatonotus*, and a Cytophagia bacterium, *Aureibacter* (Carney et al. in review). It is interesting that simultaneous crashes would occur in adjacent ponds but due to very different types of biocontaminants. In this case, microbiome analysis was key to elucidating the difference, as microscopy could only verify the presence of the rotifer in one of the raceways.

7.3.2. *Fungal parasites*

Fungal parasites are particularly insidious in algal culture. While acute fungal infections cause obvious rapid pond crashes, chronic infections cause reduced productivity over longer time periods (Carney et al. in press Algal Res). In natural freshwater environments chytrids are well known to parasitize microalgae at infection rates that exceed 90% causing severe biomass losses (Kagami et al. 2007). Chytrid infections of green algae have been described within cultivation systems as well (both open and closed) and can severely and rapidly reduce algae productivity (Fott 1967, Hoffman et al. 2008). For example, only 3 days was required for a chytrid parasite to infect 100% of algae cells in a *Haematococcus* culture (Hoffman et al. 2008).

In a prototype enclosed photobioreactor at a wastewater treatment plant in California, we analyzed a potential crash of a freshwater green alga growing in wastewater effluent that had been chlorinated and dechlorinated (Carney et al. in press Algal Res). Microbiome anlaysis revealed a small spike in the proportion of *Rhizophydium*, a known algal parasite, that correlated with a decrease in algae biomass. After a few days, this chytrid was replaced by another chytrid that was saprobic (i.e., feeding on dead algae biomass). This change in chytrid community was consistent with the end of an infection period when dead algal biomass would accumulate.

Recently, a new member of Cryptomycota, *Amoeboaphelidium protococcarum*, was discovered infecting the green alga, *Scenedesmus*, in commercial ponds in New Mexico (Letcher et al. 2013). The abundance of this aphelid was inversely correlated with that of the algae, a potential disaster considering the parasite was previously unknown. It was isolated and cultured and finally a multi-gene analysis was used to place the parasite within the Cryptomycota. Sister taxa included other parasites that had infected algae ponds.

7.3.3. *Pathogenic bacteria*

The role of bacteria in algae dominated communities is not straightforward. Bacteria are reported as both beneficial (Cole 1982, Kazamia et al. 2012) and pathogenic (Cole 1982, Fukami et al. 1997). Deciphering which strains, or which strains under which conditions, are pathogenic will be important for algae cultivation management. *Bdellovibrio*-like bacteria have been reported to lyse the green algae, *Chlorella* (Cole 1982, Coder and Starr 1978). We detected this genus in an

enclosed photobioreactor growing freshwater green algae (Carney et al. in press Algal Res). Genera from the group *Cytophagia* may also be able to infect algae and are known to attach to, secret lytic substances to dissolve cell wall, and lyse cyanobacteria (reviewed by Rashidan and Bird 2001). These strains are quite common during natural cyanobacterial bloom formation (Rashidan and Bird 2001). We have repeatedly detected cytophage sequences in declining green algae cultures (Carney et al. in press Algal Res, Carney et al. in review) and it is possible that they have lytic effects on more than just cyanobacteria.

7.6. Molecular diagnostic driven solutions to contamination

Once detected by classical or more advanced monitoring methods, a rapid response is usually required in order to reduce or eliminate the contaminant. Molecular techniques have the potential to provide accurate contaminant identification and quantification informing the an efficient and targeted response. Here we describe some of the most common responses in use today as well as some novel approaches that are currently in development. More detailed information on algae crop protection is presented in Chapter 6 of this volume (McBride)

At laboratory and small pilot scales of algae biomass production, it is possible to limit contamination by utilization of axenic cultures starter cultures, sanitization of cultivation systems, filtration or chemical sanitization of source water, and chemical treatment of mass cultures when contamination is observed. However these procedures unlikely to be economically viable at the scales required for commercial production and the general application of antibiotics and disinfectants to aquaculture feed and facilities has led to rapid development of drug-resistant pathogens, leading to many unmanageable outbreaks (Schwarz et al. 2001, Akinbowale et al. 2006).

As a consequence, there is significant effort using both classical strain selection and molecular biological techniques, to develop algal strains that have enhanced resistance contaminants may be required (Georgianna and Mayfield 2012), adding to R&D costs. Due to these contamination issues, some researchers have concluded that the cultivation of algae in open ponds may not be viable and instead closed-system photobioreactors will have to be relied upon (Ugwa et al. 2008, Rios et al. 2013). Pending significant technological breakthrough this would more than double the cost of algae biofuel (Davis et al. 2011, Gao et al. 2012).

7.4.1. *Salvage harvest*

The simplest, and perhaps least satisfying response to an incipient crash is to harvest the biomass once contaminants are detected and prior to a total loss of the desired product. Because of the potential for rapid biomass loss, early detection can be critical. Although this may save costs in terms of production time, the harvested product may have suboptimal algal density and lower concentrations of the desired product. Additionally, contamination may carry over to future cultures if the cultivation system is not sufficiently sterilized after salvaging the infected culture.

7.4.2. *Chemical agents*

Bio-contaminates in algae cultivation systems are commonly treated with chlorine, which deleteriously affects the entire community. Although the algae can recover productivity, recovery takes several weeks. Further, chlorine application at a large scale may be cost-prohibitive. Clearly, more targeted protection is needed. The fungicide, thiophanate-methyl, has been used successfully to protect amphibians from chytrid infections (Hanlon et al 2012) and may be promising for aquatic algae cultures (e.g., Shurin et al. 2013). However, the cost of applying antifungal agents at the large scale may be cost-prohibitive and it may be necessary to limit its application to smaller scale starter cultures while taking extra precaution to avoid contamination at larger scales. Microalgae may also have natural antibacterial abilities (Kellam and Walker 1989) that may be exploited in a mixed-algae production system where protective properties of one algae benefit the target species. Lastly, with increased understanding of the life cycles of specific biocontaminants, more effective chemical control can be developed. For example, chlorine treatment or nutrient addition could be timed in order to disfavor certain vulnerable life stages of the contaminant.

7.4.3. *Biological control*

Bio-control is widely used in terrestrial agriculture and on public lands to manage unwanted pests ranging from invasive plants to mammals. Bio-control of plant pathogens has been gaining momentum (36% of existing bio-control agents have been developed in the last 5 years) and is often preferred over chemical means of control. Chemical residues may hinder downstream product processing, regulation of agrochemical has become increasingly restrictive, and target pests are likely to develop chemical resistance to pesticides (Fravel 2005). Organisms that prey on and parasitize microalgae have their own set of predatory parasites, termed hyperparasites. Hyperparasites that infect many important parasites of algae, have potential as a novel biological control mechanism in algal production ponds. Evidence suggests hyperparasite infections do not kill their fungal host but instead reduce their reproductive success by efficiently co-opting the cytoplasm of infected cells and thereby indirectly reducing infection rates of algae (Gleason et al. 2012). Examples of such relationships are common in freshwater environments and a marine example has been described (reviewed by Gleason et al. 2012). In addition, metagenomic evidence suggests hyperparasites are common in marine communities (James and Berbee 2012). The host range of hyperparasites has been determined to be narrow with each species of hyperparasite infecting only closely related species. It is feasible that a dried hyperparasite stock could be used to inoculate fungal-infected algal production ponds in order to control the fungal parasite. In fact, fungal species that have resting spores as part of their life cycles are noted as being easy to formulate as bio-control agents because they are easier to ship and have lower risk of contamination (Fravel 2005).

7.4.4. *Disruption of quorum-sensing in bacteria*

Quorum sensing is the means by which bacteria regulate their own gene expression through intra-species chemical signaling and has been linked to disease induction in aquatic populations (reviewed by Natrah et al. 2011). In nature, some organisms, including microalgae, appear to have the ability to disrupt quorum-sensing, thereby regulating pathogen-induced disease. For example, when algal cultures attain certain densities, the pH of the culture medium is increased to levels where quorum sensing is inhibited for certain species of bacteria (Yates et al. 2002).

Although, the effects on quorum sensing have only rarely been studied for microalgae (and then only in freshwater species), it is very common in seaweeds (macroalgae) (Natrah et al. 2011). Preliminary work reports only stimulatory effects of some green microalgae on bacterial quorum sensing (see Natrah et al. 2011).

7.4.5. *Community engineering, selective breeding and genetic modification*:
Several additional approaches may be used to increase algal defenses against predators and pathogens. The high amount of diversity among the algae suggests great potential for selective breeding and strain selection in order to maximize specific properties, including resistance to culture contamination. Just as the vast majority of terrestrial agricultural crops are domesticated strains, optimal algae production may depend on this approach (Georgianna and Mayfield 2012). Some researchers focus their efforts one step further on synthetic biology and genetically modifying algal genomes to perform more optimally. For example, utilizing quorum-sensing as described above to inhibit bacterial growth etc. (section 7.4.4. *Disruption of quorum-sensing in bacteria*), may entail modifying specific algal pathways that drive production of molecules directing intracellular communication (reviewed by Georgianna and Mayfield 2012). Another, more ecologically driven approach to building defenses is community engineering. For this, microalgal diversity, species composition and environmental conditions are managed in order to optimize species complementarity and resistance to contamination, among other culture characteristics (Shurin et al. 2013). Preliminary work suggests algae species richness affects grazer survival, however more investigations are needed to identify ideal algae consortiums (Shurin et al. 2013).

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Figure 1 Map of SSU rRNA gene showing variable regions (Esherichia coli nucleotide numbering.

Figure 2 Map of rRNA transcript structure showing ITS regions

Figure 3 Map variable regions in LSU gene

Fig. 1

SSU rRNA

Fig. 2

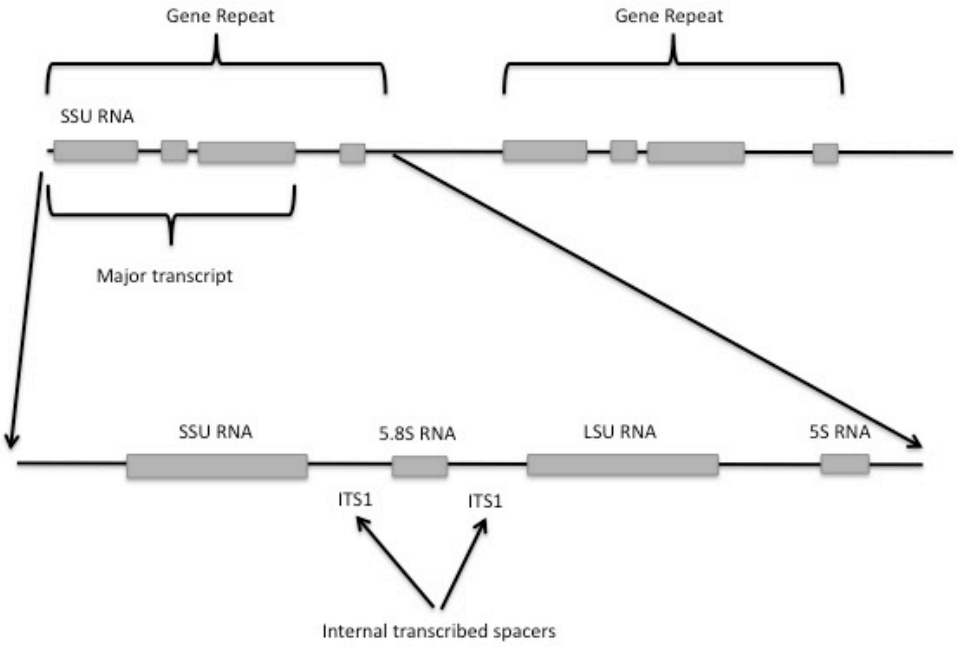
Gene Repeat
Gene Repeat
SSU RNA
Major transcript
SSU RNA
5.8S RNA
LSU RNA
5S RNA
ITS1
ITS1
Internal transcribed spacers

Fig. 3