

# Chapter 2

## Media and Conditions for the Growth of Halophilic and Halotolerant Bacteria and Archaea

Mark A. Schneegurt

### Introduction

An awareness of haloarchaea has existed since ancient times, with published descriptions of “red waters” associated with salt mining, the “red heat” of salted hides, and the “reddening” of salted fish (Bass-Becking 1931; Kurlansky 2002). For a society without refrigeration, the economic impact of codfish deterioration garnered particular attention, with Farlow (1878) oft cited as the first to publish on what were presumably haloarchaea. The early growth media of Eddington (1887) and Le Dantec (1891) reflected natural high-protein substrates, using beef peptone, gelatins, and fish broths, solidified with agar, flour, or bread paste. While some early studies used pieces of fish soaked in various brines (Høye 1908; Klebahn 1919; Harrison and Kennedy 1922), many included ground cod or a cod broth, or media based on beef bouillon or beef gelatin (Beckwith 1911; Bitting 1911; Becker 1912; Kellerman 1915; Clayton and Gibbs 1927; Velu 1929). Milk was introduced as a preferred organic constituent by Bitting (1911) and Kellerman (1915), but was popularized by Lockhead (1934). Rice flour, wheat flour or whole rice grains often were used as gelling agents (Clayton and Gibbs 1927; Robertson 1931; Boury 1934; Gibbons 1937). Silica gel was suggested to reduce organic content of solidified media (Hanks and Weintraub 1936; Moore 1940, 1941). It was recognized that alkaline culture conditions were useful for growing certain halophilic microbes (Stather and Liebischer 1929) and that halophilic obligate anaerobes could be grown on a cooked meat medium (Baumgartner 1937). The seminal paper of Harrison and Kennedy (1922) focused on the difficulties of growing the organisms responsible for red discolorations on salted fish, trying many media recipes including those based on cider, milk, broths, sugars, and potatoes. While the red organisms proved difficult to isolate, as an aside, the paper discusses a broad diversity of non-red halophilic organisms that were more easily isolated on these media.

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M. A. Schneegurt (✉)

Department of Biological Sciences, Wichita State University, Wichita, KS 67260-0026, USA  
e-mail: mark.schneegurt@wichita.edu

The influential work of Lockhead (1934) provided three recipes, a medium with skim milk and salt, a medium with codfish broth, peptone, glycerin, and salt, and a medium with beef extract, yeast extract, peptone, starch, and salt. These were widely used and included in the studies of Gibbons (1937), and of Hess (1942), who also used a medium based on Irish moss extract (carrageenan), seawater, and solid gypsum. Weber (1949) advanced the Lockhead media, and this became the basis of recipes reflected in more contemporary media (Dussault and LaChance 1952; Katznelson and Lockhead 1952). Defined media recipes often trace their history to Petter (1931) whose medium included asparagine and glycine, and again to Weber (1949). These early media led to the common media types used today, developed by Sehgal and Gibbons (1960), Rodriguez-Valera et al. (1980), Tindall et al. (1980), and Vreeland et al. (1980). Initial work on Dead Sea mud by Lortet (1892) was greatly expanded through the seminal work of Volcani (1944). His wide variety of hypersaline media was designed for aerobes, fermenters, denitrifiers, methanogens, and others. These media were based on Dead Sea waters supplemented with organic sources, ranging from cellulose to paraffin to kerosene. Oren (1983a, 1983b) continued the evolution of media using Dead Sea waters.

Early literature on organisms from salted foods and solar salt interjects a running debate on the nature of adaptation to hypersaline environments. Smith (1938) reviewed the arguments, which center on whether halophilism is an evolutionary consequence or simply the adaptation of a single generation. Rubentschik (1929) and Golikowa (1930) are credited with the first distinction between halophiles and halotolerant organisms. The former require high salinities for growth, while the latter can grow in both low and high salinities. Horowitz-Wlassowa (1931) equated halophilicity with halotolerance, introducing the term “halobe” for obligate halophiles, while Hof (1935) defined halophiles as organisms that can grow at 3 M salt. Flannery (1956) defined obligate halophiles as those requiring 2 % or more salt, while facultative halophiles grow best at greater than 2 % salinity, but also grow with less salt.

An important review by Larsen (1962) outlined a scheme that has relevance today. Nonhalophiles are those microorganisms that grow best below 2 % salt. Slight, moderate, and extreme halophiles are those that grow best in media containing 2 to 5 %, 5 to 20 %, and 20 to 30 %, respectively. Kushner (1968) then distinguished between obligate moderate halophiles and obligate extreme halophiles, which require 0.5 to 3.5 M and 3.0 M to saturated salinities, respectively. Kushner later (1978) added a definition for borderline extreme halophiles that grow best at 0.5 to 2.5 M salinity. These definitions were codified in the last edition of this review volume, but Kushner (1993) expressed concern about the use of “grows best” in the definitions of Larsen, as this could be misleading, suggesting broad optima even for extreme halophiles. Thus, halophiles require a minimum salinity for growth. Halotolerant organisms then are nonhalophiles that can grow at high salinities. Facultative halophiles require high salt only under certain environmental conditions.

For this review, halophile will be used to describe any organism that requires salinities higher than typical seawater for growth. Organisms that do not require high salt, but can grow at salinities above that of seawater will be considered halotolerant. It

can be argued that many marine organisms are slight halophiles under Kushner's scheme. The current review does not include a discussion of media for marine organisms that do not exhibit a greater degree of halotolerance or halophilicity. A wide variety of artificial seawater preparations are available (Zobell 1946; Provasoli et al. 1957). The media discussed here typically contain no less than 5 % salinity. This review also focuses only on media for halophilic and halotolerant bacteria and archaea. Saline media for eukaryotic algae (McLachlan 1960; Ben-Amotz and Avron 1983), fungi (Pitt and Hocking 1985; Gunde-Cimerman et al. 2009), or protists (Post et al. 1983; Esteban and Finlay 2003) are not discussed.

## Growth Media

### *General Comments*

The preparation and use of hypersaline media presents challenges unique to high salinities, as well as, some of the same concerns inherent with any microbial culture system. Choosing appropriate media and growth conditions is important and published media are typically associated with a particular microbial genus or species. As with other microbial discovery research, when working with environmental samples harboring communities of novel microbial populations, the media and growth conditions chosen will enrich for certain populations and not others. As a general rule, halotolerant and moderately halophilic bacteria are found at lower salinities in the environment and are cultured at room temperature with perhaps 10 % salinity. Extreme halophiles are predominantly archaea and are cultured at warmer temperatures (37 °C) with salinities of 20 % or more. The segregation of isolates into these classes using this enrichment scheme is not thorough, but the trends support this strategy. Specialized media and conditions are used to enrich for microbes from specific biogeochemical guilds, anaerobes, and alkaliphiles.

Hypersaline media can be divided into complex media that include organic components for which exact chemical formulae are not known and defined media where all components can be described by chemical formulae. There is a wide range of organic ingredients used for hypersaline media, the most popular of which are yeast extract, peptone, tryptone, and casamino acids (v.i.). The predominant salt is nearly always NaCl. Additional salts are often constituted like seawater, since the bulk of hypersaline research has been done in marine solar salterns or other thalassohaline environments. Extreme halophile media often have elevated levels of magnesium, particularly for Dead Sea isolates. The source of water used for hypersaline media preparation varies, with some media based on natural waters from the sea or hypersaline lakes (Volcani 1944; Madeley et al. 1967; Oren 1983a,b; Paterek and Smith 1985; Franzmann et al. 1987; Yu and Kawamura 1987; Wais 1988; Bertrand et al. 1990). Growth media can be prepared with tap water to provide trace minerals or with distilled water to avoid potential toxins. If phosphate is included in a medium with

substantial magnesium or calcium levels, it is commonly prepared as component solutions that are mixed after autoclaving to avoid precipitates. Other additives, mainly vitamins, are typically filter-sterilized and added to cooled media after autoclaving. One must take care when preparing media with very high salt concentrations to adjust the amount of water used to dissolve the salts such that the initial solution is not saturated and such that the final volume is not exceeded during preparation. Standard precautions used for the preparation of anaerobic media also apply to hypersaline media.

Microbial growth in media of high salinity is often slow, so it is not unusual to maintain cultures for weeks rather than days. Evaporation from liquid cultures, especially shake-flasks at elevated temperatures, can be problematic and lead to changes in salinity with time and even salt precipitation. Agar plates already present a relatively dry environment, so the addition of high salt exacerbates potential limitations. It is prudent to wrap plates in plastic paraffin film to retain moisture. It is advisable to store plates in a moist chamber, as first suggested by Le Dantec (1891). This can be as simple as sealed plastic bags (Post 1977; Rodriguez-Valera et al. 1985) or a plastic tub with a secure but unsealed cover, in which an open beaker of water or brine is kept (Caton et al. 2004). Plates will remain hydrated longer and are less likely to begin crystallization.

Another general consideration when working with hypersaline cultures is that the appearance and growth habit of microbial isolates can change depending on salinity. For *Halobacterium* and some halococci, red pigmentation is increased at higher salinities (Kushner 1993). In contrast, *Haloferax* may be more highly pigmented at lower salinities and colorless at high salinities (Rodriguez-Valera et al. 1980; Kushwaha et al. 1982). Colonies that are less highly colored, appearing cream or yellow, can exhibit more subtle changes in color at different salinities. Colonies may become smaller or mucoidy with increasing salinity. Cells may be smaller at higher salinities, often falling in the submicron range, making staining protocols more difficult. In addition, classic staining and biochemical tests have to be modified for higher salinities and may not be as consistent. For instance, carbon substrate utilization analysis using the Biolog system can give unreliable results in hypersaline solutions. Responses also may change with nutritional needs at different salinities (Litzner et al. 2006) and with changes in active transport systems (Kushner and Kamekura 1988). Variations in envelope characteristics and lipid composition are seen at higher salt concentrations, with increases in negatively charged phospholipids (Vreeland 1987; Kushner and Kamekura 1988).

## ***Media Composition***

Modern hypersaline media can trace their roots to a handful of influential media recipes, readily modified to meet specific needs. The recipes for several hypersaline media directed at haloarchaea are given in Table 2.1, while a group of common media for moderate halophiles and halotolerant bacteria is given in Table 2.2. Complex media from the Gibbons laboratory (Brown and Gibbons 1955; Abram and Gibbons

**Table 2.1** Compositions of common extreme halophile media

| Component                                       | Medium composition (g L <sup>-1</sup> ) |      |      |     |     |     |
|---|---|------|------|-----|-----|-----|
|   | A                                       | B    | C    | D   | E   | F   |
| NaCl  | 250                                     | 125  | 234  | 250 | 220 | 200 |
| KCl   | 2                                       |      | 6    | 2   | 5   | 4   |
| K <sub>2</sub> SO <sub>4</sub>                  |   | 5    |      |     |     |     |
| KNO <sub>3</sub>                                |   |      |      |     | 1   |     |
| K <sub>2</sub> HPO <sub>4</sub>                 |   |      |      |     |     | 0.5 |
| (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> |   |      |      |     |     | 1   |
| MgSO <sub>4</sub> ·7H <sub>2</sub> O            | 20                                      |      | 29   |     | 10  | 20  |
| MgCl <sub>2</sub> ·6H <sub>2</sub> O            |   | 50   | 19.5 | 20  |     |     |
| CaCl <sub>2</sub> ·6H <sub>2</sub> O            |   | 0.12 | 1.1  | 0.2 | 0.2 |     |
| NaBr  |   |      | 0.8  |     |     |     |
| NaHCO <sub>3</sub>                              |   |      | 0.2  |     |     |     |
| FeCl <sub>2</sub>                               | 0.023                                   |      |      |     | *   |     |
| Na-citrate                                      | 3                                       |      |      |     | 3   |     |
| Casamino acids                                  | 7.5                                     |      |      |     |     |     |
| Yeast extract                                   | 10                                      | 5    | 5    | 5   | 1   |     |
| Tryptone/peptone                                |   | 5    |      | 5   | 5   |     |
| Glycerol  |   |      |      |     |     | 2.5 |
| Pyruvate  |   |      |      |     |     | 2.5 |

A, Sehgal and Gibbons 1960; B, Mullakhanbhai and Larsen 1975; C, Rodriguez-Valera et al. 1980; D, Tomlinson and Hochstein 1972; E, Caton et al. 2004; F, Vreeland et al. 2002. (Adapted from Kushner 1993).

\*Includes trace minerals with iron.

**Table 2.2** Compositions of common moderate halophile media

| Component  | Medium composition (g L <sup>-1</sup> ) |       |        |        |       |
|--|---|-------|--------|--------|-------|
|  | A                                       | B     | C      | D      | E     |
| NaCl   | 80                                      | 98    | 29–174 | 29–174 | 80    |
| KCl  |   | 2     |        | 0.02   |       |
| MgSO <sub>4</sub> ·7H <sub>2</sub> O   | 20                                      | 1     | 0.1    | 2      | 20    |
| CaCl <sub>2</sub> ·6H <sub>2</sub> O   |   | 0.36  |        |        |       |
| NaBr   |   | 0.23  |        |        |       |
| NaHCO <sub>3</sub>   |   | 0.06  |        |        |       |
| FeCl <sub>3</sub> ·6H <sub>2</sub> O   |   | 0.001 |        |        |       |
| Na-citrate   | 3                                       |       |        |        | 3     |
| Casamino acids   | 7.5                                     |       |        |        | 7.5   |
| Yeast extract  | 1                                       | 10    |        |        | 1     |
| Tryptone/peptone   | 5                                       | 5     |        |        | 3     |
| (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>                                      |   |       | 2      |        |       |
| Fe(NH <sub>4</sub> ) <sub>2</sub> (SO <sub>4</sub> ) <sub>2</sub> ·6H <sub>2</sub> O | 0.05                                    |       |        |        | 0.005 |
| K <sub>2</sub> HPO <sub>4</sub>  | 0.5                                     |       | 3.12   |        | 7.5   |
| KH <sub>2</sub> PO <sub>4</sub>  |   |       | 0.28   | 0.01   |       |
| NH <sub>4</sub> Cl   |   |       | 2      |        |       |
| Glucose  |   | 1     | 10     | 10     |       |
| Glutamate  |   |       |        | 2      |       |
| Trace minerals   |   | yes   |        |        |       |

A, ATCC 1097; B, Caton et al. 2004; C, Forsyth and Kushner 1970; D, Kamekura et al. 1985; Vreeland et al. 1980.

**Table 2.3** Hypersaline ATCC Media Suggested for Common *Bacteria* and *Archaea*

| Target                  | ATCC medium  |
|-------------------------|--|
| <i>Bacteria</i>         |  |
| <i>Bacillus</i>         | 1659, 1660   |
| <i>Chromohalobacter</i> | 87, 1097   |
| <i>Halobacillus</i>     | 925 <sup>a</sup>   |
| <i>Halomonas</i>        | 87, 1097, 1582, 1689, 1725, 1740, 2049, 2084, 2096, 2097, 2168 |
| <i>Marinococcus</i>     | 87, 800  |
| <i>Salinibacter</i>     | 2402   |
| <i>Archaea</i>          |  |
| <i>Haloarcula</i>       | 1218, 1230   |
| <i>Halobacterium</i>    | 213 <sup>b</sup> , 1218, 1270                                  |
| <i>Haloferax</i>        | 974, 1270  |
| <i>Halorubrum</i>       | 1218, 1394, 1682, 2168, 2402                                   |
| <i>Halosimplex</i>      | 2235   |
| Selective               |  |
| Alkaline <sup>c</sup>   | 1392, 1590, 2049, 2096, 2097                                   |
| Anaerobic               | 1275, 1279, 1302, 1453   |

<sup>a</sup>Also, *Pseudomonas halosaccharolytica*.

<sup>b</sup>Also, *Halogeometricum* and *Haloterrigena*.

<sup>c</sup>Includes *Natronomonas*, *Natrialba*, and *Natronococcus*.

1960; Sehgal and Gibbons 1960) are the bases for a number of both moderately saline and extremely saline media. For instance, the *Halomonas* medium of Vreeland et al. (1980) is based on Abram and Gibbons (1960). In time, as new isolates were obtained, some haloarchaeal media were more successful after the addition of carbohydrates (Tomlinson and Hochstein 1972a, 1972b, 1976). An important class of hypersaline media uses a mixture of salts that resembles the composition of concentrated seawater (Rodriguez-Valera et al. 1980). Defined media for the extreme halophile, *Halosimplex*, has broad application (Vreeland et al. 2002). The medium of Payne et al. (1960), originally designed for isolates from Lake Magadi, is the basis of many hypersaline alkaline media (Tindall et al. 1980). More than 30 hypersaline media recipes are suggested by ATCC. ATCC media for common halotolerant and halophilic microbes are given in Table 2.3. A summary of the prevalence of specific ingredients in ATCC media is given in Table 2.4.

## Salinity

The selection of media for halophilic and halotolerant aerobic heterotrophs often is based on salinity. Media for halotolerant bacteria typically contain lower salinities than media specific for halophilic archaea. A survey of media recipes suggested by ATCC finds that nearly half have salinities between 5 % and 10 % (Table 2.4). Media with salinities above 20 % are suggested for halophilic archaea. Related *Halomonas* media have 8 % salinity (Table 2.2; Vreeland et al. 1980; ATCC 1097). A group of media used for enrichments of moderately halophilic and halotolerant bacteria

**Table 2.4** Composition of hypersaline ATCC Media. Number of media recipes containing each component are given

| Major salts                        |    | Minor salts  |    | Complex additions             |    |
|------------------------------------|----|--|----|-------------------------------|----|
| <i>NaCl</i>                        |    | <i>Iron</i>  |    | <i>Organic C</i>              |    |
| 5–10 %                             | 13 | As (NH <sub>4</sub> ) <sub>2</sub> (SO <sub>4</sub> ) <sub>2</sub> | 4  | Yeast extract                 | 20 |
| 11–19 %                            | 7  | as Cl <sub>2</sub>   | 4  | Peptone/Tryptone              | 14 |
| ≥20 %                              | 8  | as SO <sub>4</sub>   | 2  | Casamino acids                | 8  |
| <i>Magnesium</i>                   |    | <i>Calcium</i>   |    | Citrate                       |    |
| as citrate                         | 1  | as Cl <sub>2</sub>   | 11 | Glucose                       | 5  |
| as SO <sub>4</sub>                 | 14 | as SO <sub>4</sub>   | 1  | Glutamate                     | 2  |
| as Cl <sub>2</sub>                 | 11 | <i>Trace</i>   |    | Glycerol                      | 2  |
| as NO <sub>3</sub>                 | 1  | HCO <sub>3</sub>   | 6  | Soluble starch                | 1  |
| >50 g/L Mg salt                    | 2  | Br   | 4  | Succinate                     | 1  |
| <i>Sulfate</i>                     |    | B  | 4  | Pyruvate                      | 1  |
| as Mg                              | 20 | Cu   | 4  | pH buffer                     | 1  |
| as K                               | 2  | Mn   | 4  | Vitamins                      | 4  |
| <i>Phosphate<sup>a</sup></i>       |    | Mo   | 4  | Complex anaerobe <sup>b</sup> | 3  |
| as Na                              | 1  | Zn   | 4  |                               |    |
| as K                               | 8  | Co   | 3  |                               |    |
| <i>Nitrogen</i>                    |    | Ni   | 2  |                               |    |
| as NH <sub>4</sub> Cl              | 4  |  |    |                               |    |
| as NH <sub>4</sub> SO <sub>4</sub> | 3  |  |    |                               |    |
| as NO <sub>3</sub>                 | 2  |  |    |                               |    |
| <i>Potassium</i>                   |    |  |    |                               |    |
| as Cl                              | 17 |  |    |                               |    |
| as PO <sub>4</sub>                 | 7  |  |    |                               |    |
| as SO <sub>4</sub>                 | 2  |  |    |                               |    |
| as NO <sub>3</sub>                 | 1  |  |    |                               |    |

<sup>a</sup>≥As NaHPO<sub>4</sub> and K<sub>2</sub>HPO<sub>4</sub>.

<sup>b</sup>ATCC Medium 1279, 1302, and 1453.

(*Bacillus*, *Halobacillus*, *Halomonas*, *Salibacillus*, *Salinibacter*) has approximately 10 % salinity (Rodriguez-Valera et al. 1980; Quesada et al. 1983; Caton et al. 2004). While the popular halophilic archaeal medium of Mullakhanbhai and Larsen (1975) has a salinity of 12.5 %, other popular halophilic media have 23 % or greater salinities (Table 2.1). These media support the growth of a wide range of haloarchaea including *Haloarcula*, *Halobacterium*, *Halococcus*, *Haloferax*, and *Halorubrum*.

For some media, NaCl is supplemented with other salts in an effort to mimic the composition of concentrated seawater. These media are best suited for organisms from solar salterns fed with seawater or other thalassohaline waters and soils. The most popular of these media are based on Rodriguez-Valera et al. (1980) that derives its salt mixture from earlier work by Subov (1931). It includes 2 % KCl and lesser concentrations of CaCl<sub>2</sub>, NaBr, and NaHCO<sub>3</sub>.

It is not uncommon for media to be developed using natural saline waters or salt mixtures obtained from the natural sources where microbial specimens were collected (Eimhjellen 1965; Madeley et al. 1967; Mathrani and Boone 1985; Yu and Kawamura 1987; Franzmann et al. 1988; Wais 1988). Even the popular *Halomonas*

medium of Vreeland et al. (1980) originally included solar salt from a study site in the Netherlands Antilles. A wide range of media developed by Volcani (1944) is based on waters from the Dead Sea diluted to different salinities and then extended by Oren (1983a,b). Filtered seawater or salt plains brine were effective bases for f/2 medium (Guillard and Ryther 1962) in growing halotolerant cyanobacteria and algae (Garcia-Pichel et al. 1996; Henley et al. 2002; Kirkwood and Henley 2006), as was Hamelin pool water as a basis for BG-11 medium (Goh et al. 2010). Wais (1988) was successful in isolating haloarchaea using natural brines from thalassohaline lagoons and suggested that conventional media may be less effective for enrichment cultures.

The early work of Schoop (1935) replaced NaCl in growth media with KCl,  $\text{KNO}_3$ ,  $\text{Na}_2\text{CO}_3$ , and  $\text{NaNO}_3$ . None of these replacements were suitable for obligate halophiles, but facultative halophiles grew with these substitutions. Based on the medium of Sehgal and Gibbons (1960; supplemented with  $\text{FeCl}_2$ ), studies on the extreme halophile *Halobacterium cutirubrum* have examined the effects of replacing NaCl with other salts by observing cell morphology and leakage (Abram and Gibbons 1961; Boring et al. 1963). At salinities below 1.5 M, significant cell lysis occurred, while at salinities below 3.5 M NaCl, cell morphology was altered. In media containing 0.1 M NaCl, cells remained intact with 1–2 M concentrations of  $\text{CaCl}_2$ ,  $\text{MgCl}_2$ , Na-acetate, or  $\text{Na}_2\text{S}_2\text{O}_3$ . Growth can be supported in media where NaCl is partially replaced by other solutes, such as KCl for haloarchaea (Brown and Gibbons 1955; Gibbons 1969; Kushner 1985) or sucrose for halotolerant bacteria (MacLeod 1965; Adams et al. 1987). However,  $\text{Na}^+$  seems to be a broad requirement of haloarchaea and halotolerant bacteria. Generally for the halophilic archaea, NaCl is needed at a concentration of at least 1.5 M, in addition to other salts (Mohr and Larsen 1963).

Suitable substitutes for *Halobacterium volcanii* were shown to be NaBr,  $\text{NaNO}_3$ ,  $\text{MgCl}_2$ , and KCl (in that order), but not  $\text{Na}_2\text{SO}_4$  (Mullakhanbhai and Larsen 1975). The haloarchaea seem to require  $\text{Cl}^-$  as well as  $\text{Na}^+$  ions for growth (Brown and Gibbons 1955). *Halomonas elongata* was found to grow well with NaCl replaced by NaBr or  $\text{NaNO}_3$ , but not NaI or  $\text{Na}_2\text{SO}_4$ , while *Deleya halophila* could use NaBr,  $\text{Na}_2\text{SO}_4$ , and  $\text{Na}_2\text{S}_2\text{O}_3$ , but not other sodium salts (Vreeland and Martin 1980; Quesada et al. 1987). *Vibrio costicola* was able to grow on NaBr,  $\text{NaMO}_4$ ,  $\text{NaPO}_4$ , and  $\text{Na}_2\text{SO}_4$ , with weak growth on LiCl, KCl, and  $\text{MgCl}_2$  (Flannery et al. 1952). In a study of 168 halophilic bacterial isolates, 75 strains required NaCl, while 21 strains grew well on media with 1–4 M KCl (Onishi et al. 1980). A wide variety of salts at 1 M concentration supported growth of *Micrococcus varians* including KBr, KCl,  $\text{KNO}_3$ , RbBr, RbCl, and a number of Na salts (Kamekura and Onishi 1982). In this case, media made with KI or NaI showed growth after a long lag period, and strains capable of growth with high concentrations CsCl and LiCl were isolated. As discussed below, some microorganisms from oligohaline environments may be osmotolerant or osmophilic, but naturally depend on salts other than NaCl.



## Magnesium

Requirements for Mg salts are broad among halotolerant and halophilic microorganisms. While  $\text{Mg}^{++}$  is a common component of microbiological media, haloarchaea are generally considered to require higher concentrations of  $\text{Mg}^{++}$  for growth. However, not all do and the growth of some halophilic and halotolerant microbes is inhibited by higher  $\text{Mg}^{++}$  concentrations (Soliman and Trüper 1982; Juez 1988). *Halobacterium cutirubrum* required at least 0.1 M  $\text{Mg}^{++}$  for growth, helping cells maintain normal morphology at lower (2.5 M) salinities (Boring et al. 1963). While slow growth was observed at  $\text{Mg}^{++}$  concentrations of 0.01–0.025 M, maximum growth occurred in the range of 0.1–0.5 M  $\text{Mg}^{++}$  for *Halobacterium halobium*, *Pseudomonas cutirubra*, *P. salinaria*, and *Sarcina littoralis* (Brown and Gibbons 1955). Magnesium salts of chloride, nitrate, and sulfate were equally effective.

A special case, are microbes isolated from the Dead Sea. These waters have a  $\text{MgCl}_2$  concentration of 1.1–1.5 M, in addition to 1.7 M NaCl. The early work of Volcani (1944) used Dead Sea water for a variety of media. Isolates from the Dead Sea are typically grown in media containing 0.6–1.2 M  $\text{Mg}^{++}$ , with *Halobacterium sodomense* shown to require high  $\text{Mg}^{++}$  concentrations (Oren 1983b). Calcium could partially satisfy this requirement.

The common haloarchaea media of Table 2.1 include 2–5 % Mg salts, as chlorides or sulfates. *Halomonas* media also contain 2 % Mg salts, while other common halotolerant bacteria media contain lower amounts (Table 2.2). A survey of media from ATCC (Table 2.4) shows that the chloride and sulfate salts are most popular, with only one medium using the nitrate salt. Only 2 of the 28 hypersaline media contained more than 5 % Mg salts.

## Potassium

KCl is typically added up to 2 %, to media that mimic concentrated seawater (Rodriguez-Valera et al. 1980; Caton et al. 2004; Caton et al. 2009), and is likely a component of media based on natural brines. *Halobacterium halobium*, *Pseudomonas salinaria*, *P. cutirubra*, and *Sarcina littoralis* failed to grow in media that did not contain  $\text{K}^+$  (Brown and Gibbons 1955). Maximum growth was seen at 1–3 mM, but was not inhibited at 3 M KCl. The  $\text{K}^+$  requirement could not be filled by  $\text{NH}_4^+$ ,  $\text{Cs}^+$ , or  $\text{Li}^+$ , but higher concentrations of  $\text{Rb}^+$  supported growth, as did the addition of ash from yeast extract. *Halobacterium* did not grow below 12.5 mg  $\text{L}^{-1}$   $\text{K}^+$  and grew best at 1 %  $\text{K}^+$  (Gochnauer and Kushner 1969). A survey of ATCC media (Table 2.4) shows that most use the chloride salt, while others use a phosphate salt, likely to provide pH control. Media containing nitrate or sulfate salts also have been suggested. Common haloarchaea media contain between 0.2 and 0.5 % K salts (Table 2.1). The potential for K salts to partially or completely replace Na salts in media for halophilic and halotolerant microbes is discussed above.

## ***Sulfur, Phosphate, and Nitrogen***

Complex media typically rely on organic materials as sources of sulfur and nitrogen. In defined media, these can be supplied as inorganic chemicals or as amino acids. Nitrogen can be provided as  $\text{NH}_4\text{Cl}$ ,  $\text{NaNO}_3$ , or  $(\text{NH}_4)_2\text{SO}_4$  (Table 2.4). The amino acids asparagine, cysteine, glutamate, glutamine, and histidine have been used (Flannery and Kennedy 1962; Onishi et al. 1965; Forsyth and Kushner 1970; Grey and Fitt 1976; Yu and Kawamura 1987; Kauri et al. 1990). Sulfate often appears as a counterion for Ca, Fe, Mg, N, and K salts, while phosphate is a counterion in K and Na salts. Aerobic, chemolithoautotrophic, sulfur-oxidizing *Thiohalobacter* uses thiocyanate as an electron donor (Sorokin et al. 2010).

## ***Minor and Trace Salts***

Iron is often added to both defined and complex hypersaline media as chloride, citrate, or sulfate salts, or as a double salt with ammonium sulfate. Typically it is not provided in the chelated forms common for plant media. The influential medium of Sehgal and Gibbons (1960) is often supplemented with  $\text{FeCl}_2$  (Boring et al. 1963; Kushner and Bayley 1963). Iron has been shown to be essential for growth of halophilic and halotolerant microbes at concentrations similar to those used for other bacteria and archaea (Brown and Gibbons 1955).

Calcium salts are included in mixtures that mimic seawater salts and in defined media, mainly  $\text{CaCl}_2$ . It can be found at 0.4 M in Dead Sea waters. No specific studies address requirements for calcium or the trace minerals. Seawater mimics include  $\text{NaBr}$  and  $\text{NaHCO}_3$  in addition to  $\text{CaCl}_2$  (Rodriguez-Valera et al. 1980; Caton et al. 2004; Caton et al. 2009), at low concentrations (<0.5 %). Trace mineral mixtures can include B, Co, Cu, Mn, Mo, Ni, or Zn, but generally no trace minerals are added to hypersaline media. With such high salinities, it can be expected that trace mineral requirements will be filled by contaminants in the laboratory chemicals. Trace minerals are likely present in media made from natural brines, tap water, or solar salts.

## ***Organic Components***

Work with halophiles began with organisms found on salted fish and meats, so early media recipes focused on protein and amino acid sources of C, including ground fish, peptone, and skim milk (Le Dantec 1891; Beckwith 1911; Kellerman 1915; Klebahn 1919; Stuart et al. 1933; Lockhead 1934; Stuart 1940). For the extreme halophiles, it was thought that carbohydrates were not good C sources. This paradigm changed with the isolation of *Halobacterium saccharovorum* that grows on a range of simple carbohydrates (Tomlinson and Hochstein 1972a, 1972b, 1976). In addition, *Haloarcula*

*marismortui*, *Haloarcula trapanicum*, *Haloarcula vallismortis*, and *Halobacterium sodomense* grow well on a variety of carbohydrates (Juez 1988; Oren 1994). Some carbohydrates can support the growth of *Haloferax mediterranie*, *Haloferax volcanii*, and *Natronobacterium pharaonis*. Essentially no growth is obtained with carbohydrates for *Halobacterium cutirubrum*, *Halobacterium salinarum*, and *Halococcus morrhuae*. This last species is considered to be equivalent to the earliest haloarchaea studied, formerly designated as *Sarcina litoralis* and *Micrococcus morrhuae* (Juez 1988). Halotolerant bacteria typically have wider metabolic abilities than haloarchaea, thus, many carbon sources are suitable (Ventosa et al. 1982; Quesada et al. 1983; Litzner et al. 2006).

Yeast extract is the most popular carbon source in complex media for halophilic and halotolerant bacteria and archaea (Dundas et al. 1963; Tomlinson and Hochstein 1972a,b; Post 1977; Rodriguez-Valera et al. 1980; Ventosa et al. 1982; Caton et al. 2004). It was a component of several Gibbons' media (Abram and Gibbons 1960; Sehgal and Gibbons 1960) that form the bases of modern media recipes. Yeast extract is found in 20 hypersaline ATCC media (Table 2.4) and derivatives of popular moderate halophile media (Vreeland et al. 1980; Quesada et al. 1983, 1985). Taken together, the second most broadly used carbon sources are peptones, trypticases, and tryptones, widely known from the influential work of Weber (Hof 1935; Weber 1949; Dussault and LaChance 1952; Shiio et al. 1956; Mullakhanbhai and Larsen 1975; Ishida and Fujii 1970). Casamino acids and citrate, which were included in the medium of Sehgal and Gibbons, appear in media recipes today (Post 1977; Vreeland et al. 1980). In some cases, compounds, such as carbohydrates or glycerol are the main carbon sources, but yeast extract or another amino acid source is added in low quantities (Forsyth and Kushner 1970; Ducharme et al. 1972; Tomlinson and Hochstein 1972a,b; Mullakhanbhai and Larsen 1975). These complex ingredients also can supply some mineral nutrients and complex growth factors such as vitamins.

Glucose is the most common simple carbohydrate added to complex hypersaline media (Forsyth and Kushner 1970; Rodriguez-Valera et al. 1980; Quesada et al. 1983; Oren 1986; Caton et al. 2004). Aliphatic and aromatic hydrocarbons also can serve as carbon sources (Bertrand et al. 1990; Kulichevskaya et al. 1992; Nicholson and Fathepure 2004, 2006; Al-Mailem et al. 2010). Glycerol can be used for halotolerant bacteria (Chan and Leung 1979; Vreeland and Martin 1980). Succinate and glycerol have been used for *Haloferax* (Mevarech and Werczberger 1985; Kauri et al. 1990) and acetate for *Halobacterium* (Boring et al. 1963). Mixtures of acetate, glycerol or pyruvate are used in defined media for *Halosimplex* (Vreeland et al. 2002). Media for microbes isolated from foods can contain related foodstuffs as components (Clayton and Gibbs 1927; Kono and Taniguchi 1960; Ōmata et al. 1961). A medium designed using only household materials and foodstuffs is suitable for inexpensive secondary science classroom laboratory activities (Schneegurt et al. 2004).

It should be pointed out that there have been issues in the past with certain brands of peptone (Kamekura et al. 1988). Apparently there was contamination with bile salts (glycocholic acid and taurocholic acid) in some batches, at

concentrations high enough to lyse sensitive haloarchaea such as *Haloarcula*, *Halobacterium*, *Haloferax*, and *Natronobacterium*, while not affecting *Halococcus* and *Natronococcus*. The damaging effects of bile salts had been suggested earlier (Dussault 1956). It seems that carbohydrates offer some protection from bile salt contaminants (Oren 1990). Oleates and stearates at 0.5 % or detergents also appear to lyse haloarchaea (Bertullo 1960–1961; Abram and Gibbons 1961).

Defined hypersaline media generally include amino acids and several amino acids appear to be required by certain microbial species (Petter 1931; Dundas et al. 1963; Onishi et al. 1965; Ducharme et al. 1972; Grey and Fitt 1976; Kamekura et al. 1985; Plakunov and Lobyreva 1985; Lobyreva et al. 1987). Amino acids are often added to meet these needs or as a N source, while C is supplied as carbohydrates or glycerol (Katznelson and White 1950; Onishi et al. 1965; Grey and Fitt 1976).

### ***Other Components***

Vitamins and other growth factors have been tested for their ability to stimulate growth in hypersaline media. These may be supplied with the addition of yeast extract (Kauri et al. 1990). Some vitamins are useful in *Halobacterium* and *Haloferax* medium, including biotin, thiamine, folate, and B<sub>12</sub> (Onishi et al. 1965; Gochnauer and Kushner 1969; Franzmann et al. 1988; Kauri et al. 1990), and some vitamins appear in halotolerant bacteria media (Flannery 1955; Chan and Leung 1979). Nucleic acid bases did not have much effect on growth (Katznelson and Lockhead 1952) and are rare media components (Onishi et al. 1965).

Antibiotics have been used for the purpose of selecting for particular organisms in enrichment or maintenance cultures. Penicillin is most popular, but ampicillin and streptomycin have been used (Torreblanca et al. 1986; Montero et al. 1988; Wais 1988; Kulichevskaya et al. 1992). A combination of penicillin G, erythromycin, and cycloheximide were used to select for archaea at different pHs from subzero hypersaline methane seeps (Niederberger et al. 2010). Generally pH buffers are not included in neutrophile media, but have appeared in several (Tomlinson and Hochstein 1972a,b; Tomlinson et al. 1986; Tardy-Jacquenod 1998). The pH of alkaline media is generally set by the addition of carbonates (Brown 1963; Tindall et al. 1980; Kobayashi et al. 1992; Kanai et al. 1995).

### **Specialty Media**

Most work with halophilic and halotolerant microbes has been done on strict aerobes and at neutral pH. However, a body of literature deals with various anaerobes, including fermenters and methanogens, and with organisms found in alkaline environments rich in natron. These groups require special media and growth conditions.

A detailed rendering of all of the published niche media is outside the scope of this review, but a few are discussed here.

Enrichment and maintenance media for *Clostridium*, *Halanaerobium*, and *Halobacteroides* from the Dead Sea have been presented by Oren (1983a, 1984, 1986). These are rich complex media that may include amino acid sources, glucose, glutamate, lactate, pyruvate, salts, and vitamins. These are set at a relatively low pH (6–6.5) and include components often found in media for anaerobes such as ascorbate, cysteine, resazurin, and thioglycolate. Oren also has studied the fermentative and respiratory abilities of *Halobacterium* and *Haloferax* using DMSO, TMAO, and fumarate (Oren and Trüper 1990; Oren 1991). A medium specific for a halophilic nitrate respirer, *Halobacterium dentrificans*, is rich in organic compounds and includes a pH buffer and  $\text{KNO}_3$  (Tomlinson et al. 1986).

Methanogens from hypersaline environments have been studied using complex media that typically include trimethylamine (Mathrani and Boone 1985; Paterek and Smith 1985; Zhilina 1997; Yu and Kawamura 1987; La Cono 2011). These can be organically rich media with peptone and yeast extract, and often include amino acids and vitamins. Additives to lower the reduction potential of the medium, such as cysteine and sulfides are included. Sulfate reducers are known at higher salinities and have been isolated on anoxic media (Tardy-Jacquenod 1998). The medium for *Desulfotomaculum* is moderately hypersaline at 4 % NaCl, is buffered with MOPS, and includes Na-lactate and yeast extract.

Alkaline media often mimic common hypersaline media, but the pH is set from 8 to 10 with the addition of  $\text{Na}_2\text{CO}_3$  (Payne 1960; Tindall et al. 1980; Tindall et al. 1984; Kobayashi et al. 1992; Kanai et al. 1995; Zhilina et al. 1997; Pikuta et al. 2003). These media are specific for isolates from soda lakes such as Lake Magadi in Kenya and the Wadi An Natrun in Egypt. Many of these alkaliphiles, including strains of *Natronobacterium pharaonis* and *Natronococcus occultus*, can be inhibited by  $\text{Mg}^{++}$  concentrations higher than 0.01 M (Soliman and Trüper 1982; Juez 1988), as their parent body of water is very low in soluble  $\text{Mg}^{++}$  and  $\text{Ca}^{++}$ . Anaerobic alkaline media are known, including media for isolates from Mono Lake (Guffanti et al. 1986; Blum et al. 1998; Mesbah et al. 2007). Halophilic acidophiles appear to be rare, although a growth medium set at pH 4.5 has been reported for a haloarchaeon (Minegishi et al. 2008).

Photosynthetic organisms are found in hypersaline environments with the eukaryote *Dunaliella* receiving the most attention (Ben-Amotz and Avron 1983; Rodriguez-Valera et al. 1985; Oren 2000; Henley et al. 2002; Kirkwood and Henley 2006). Media for halotolerant cyanobacteria are typically either made from seawater or artificial seawater, such as Provasoli's enriched seawater (Yopp et al. 1978; Tindall et al. 1978; Starr and Zeikus 1987; Garcia-Pichel et al. 1998). Common media such as f/2 or Chu11 are found supplemented with salts for halotolerant phototrophs (Dor and Hornoff 1985; Garcia-Pichel et al. 1996). It has been suggested that maintaining elevated temperatures (45 °C) suppresses the growth of *Dunaliella*, allowing cyanobacteria to bloom (Dor and Hornoff 1985).

## Environmental Conditions

Generally haloarchaea grow best above room temperature. Most laboratories use 37 °C (Abram and Gibbons 1960; Boring et al. 1963; Dundas et al. 1963; Ducharme et al. 1972; Matheson et al. 1976; Tomlinson and Hochstein 1976; Tindall et al. 1984; Montero et al. 1988; Yu and Kawamura 1987; Kamekura and Dyall-Smith 1995), while others work somewhat lower (35 °C) or higher (40 °C) (Kushner and Bayley 1963; Mullakhanbhai and Larsen 1975; Oren 1983b, 1986; Torreblanca et al. 1986), and optimal growth temperatures of 50 °C have been reported (Gibbons 1969; Hochstein 1988; Cayol et al. 1994, 2000; Mesbah et al. 2007). Halophilic and halotolerant bacteria are often grown at room temperature or at a slightly elevated temperature (30 °C) (Forsyth and Kushner 1970; Vreeland et al. 1980; Oren 1983a, 1986; Caton et al. 2004). Psychrotrophic and psychrophilic organisms are maintained at 5–10 °C, but can grow at or below –5 °C at high salinities (Madeley et al. 1967; Franzmann et al. 1987, 1988; Niederberger et al. 2010).

The salt response of halophilic and halotolerant microbes can be affected by growth temperature and optimal growth temperature can be affected by salinity (Ishida 1970; Mullakhanbhai and Larsen 1975; Novitsky and Kushner 1975, 1976; Vreeland and Martin 1980; Quesada et al. 1987). *Halomonas elongata* exhibited different optimal salinities and permissible ranges with changing temperatures (Vreeland and Martin 1980). Growth at higher salinities (4 M) was enhanced at 30 or 40 °C relative to 20 °C. *Planococcus halophila* required 0.5 M salt for growth when cultured above 25 °C (Novitsky and Kushner 1975). A broader study of 48 strains of *Brevibacterium* and *Flavibacterium* and from solar salt found that increasing growth temperatures from 27 to 35 °C increased minimum salinity requirements, but salinity did not affect optimal growth temperature (Ishida 1970).

Most halophilic and halotolerant microbes isolated to date are neutrophiles, growing best in media with pHs from 6.8 to 7.5 (Brown and Gibbons 1955; Abram and Gibbons 1960; Flannery and Kennedy 1962; Tomlinson and Hochstein 1972a,b; Mullakhanbhai and Larsen 1975; Vreeland et al. 1980; Mevarech and Werczberger 1985; Vreeland et al. 2002; Caton et al. 2004), although some media use pHs outside of this range (Boring et al. 1963; Kushner and Bayley 1963; Forsyth and Kushner 1970; Ishida and Fujii 1970). Anaerobes can be maintained over a similar pH range of 6–7 (Oren 1983a, 1986; Yu and Kawamura 1987). As discussed above, alkaliphilic organisms are grown at pHs of 8–10 using carbonates (or natron) to maintain the pH (Tindall et al. 1980; Kobayashi et al. 1992; Soliman and Trüper 1982; Kanai et al. 1995).

Most of the halotolerant and halophilic microbes studied to date are aerobes, although anaerobes are known (Oren 1983a, 1986; Mathrani and Boone 1985; Tomlinson et al. 1986; Zhilina 1997; Yu and Kawamura 1987). A challenge for aerobic organisms in hypersaline systems is that oxygen solubility decreases with increasing salinity, dropping by half at 10 % salinity and by over 80 % at 30 % salinity. Therefore, more vigorous aeration should be considered when culturing at higher salinities.

Illumination conditions are typically not specified in published reports on halotolerant isolates and the presumption is that microbial cultures should be maintained in the dark. Haloarchaea have been shown to contain photosensory pigments that control gene expression and responses, light-driven ion pumps, and bacteriorhodopsins that can support phototrophic growth (Sharma et al. 2007). Light has been used to drive anaerobic growth of *Halobacterium* at more than  $3 \times 10^5$  lx, near saturation for bacteriorhodopsins (Hartmann et al. 1980; Oesterhelt and Krippahl 1983). There have been reports that light can damage haloarchaeal cells as noted in colorless mutants and can inhibit respiration by reducing available ADP levels (Dundas and Larsen 1962; Oesterhelt and Krippahl 1973). Algal and cyanobacterial cultures are maintained under moderate illumination of approximately 3,000 lx (Yopp et al. 1978; Dor and Hornoff 1985; Garcia-Pichel et al. 1998; Kirkwood and Henley 2006).

## Other Salinophiles

Not all environments with high salinity are rich in NaCl. Osmotolerant microbes are typically obtained at high NaCl concentrations (halotolerant), although some are known from environments rich in sugars or other salts (Ingram 1957; Grant 2004). After the precipitation of halite in solar saltern crystallizer ponds, the remaining bitterns are typically dominated by MgCl<sub>2</sub> and KCl (Ratton 1877). It is oft cited that bitterns are “apparently devoid of life” since Javor (1982, 1983, 1984) had difficulty cultivating microbes from Guerrero Negro salterns using complex media (Sehgal and Gibbons 1960; Oesterhelt and Stoeckenius 1974). However, others report on microbes isolated from similar bitterns (Rodriguez-Valera et al. 1985; Butinar et al. 2005; Cantrell et al. 2006; Hallsworth et al. 2007). Typical hypersaline growth media were used and these were not specifically aligned with the composition of bitterns. The hypersaline anoxic Discovery Basin in the Mediterranean Sea is naturally 5 M MgCl<sub>2</sub> with low NaCl (van der Wielen et al. 2005). While *Bacillus* were isolated from this environment, the growth medium was enriched seawater that did not reflect the composition or anoxic nature of the basin (Sass et al. 2008).

Basque Lake in the Kamloops region of BC and Hot Lake near Oroville WA are athalassohaline epsomite lakes that contain precipitating concentrations of MgSO<sub>4</sub> (Epsom salt) and virtually no NaCl or other chlorides (Handy 1916; Anderson 1958; Hammer 1986; Nesbitt 2004). Organisms growing at high MgSO<sub>4</sub> concentrations might be called salinotolerant or osmotolerant, but these terms seem too broad. It is suggested that these organisms be characterized as “epsotolerant” or “epsophilic,” either growing at or requiring high concentrations of MgSO<sub>4</sub>, respectively (Crisler et al. 2012). While growth of halotolerant and halophilic organisms at high MgSO<sub>4</sub> concentrations has been reported (Markovitz 1961; Markovitz and Sylvan 1962; Boring et al. 1963; Crisler et al. 2012), no epsophilic organisms are known. One report from Basque Lake used media with 2 M MgSO<sub>4</sub>; however, the enrichment cultures were not fully described (Foster et al. 2010). Epsotolerant bacteria were isolated from efflorescences on degraded stone surfaces using media with MgSO<sub>4</sub>

concentrations as high as 25 % (Laiz et al. 2000; Mandrioli and Saiz-Jimenez 2002). An initial study (Crisler et al. 2010) of waters and sediments from Basque and Hot Lakes demonstrated good microbial growth on organically rich medium containing 2 M MgSO<sub>4</sub>, with dozens of microbial isolates capable of growth in 10 % NaCl or 10 % MgSO<sub>4</sub> media, including algae and cyanobacteria. The isolates generally appear to be broadly epsotolerant and dominated by bacteria.

## Concluding Remarks

Growth media for halophilic and halotolerant bacteria and archaea trace their composition to the high-protein fish and hides where these organisms first attracted attention. Media recipes have bifurcated into those directed at extreme halophiles and those directed at moderate halophiles and halotolerant microbes. As more isolates have been obtained from varied environments, more types of media have been modified to hypersaline variants. Development of new media and growth conditions in the future will likely be driven by the isolation of novel organisms from unique hypersaline environments.

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