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Concerning Oxidants in life support systems.

Introduction

Oxidants including Chlorine, Bromine and Ozone are commonly used in Life Support Systems for Marine Mammals. (Spotte, 1991) The goal is to reduce the potential for infectious disease and also to aid in the clarity and appearance of the water in the habitats. Their use in excess is known to cause injury but it has been thought that with careful use they can be safe. Recent discoveries concerning the biological effects of these compounds and the by-products produced by their use brings the safety of their use into question. Discussions of their use in water disinfection generally assume minimal organic matter in the water. This may not be the case in water where animals are living. These compounds reduce or eliminate biological filtration so organic and nitrogenous wastes can build up in life support systems increasing the production of by-products of disinfection (BPDs).

By-Products of Disinfection or BPDs are produced when water containing organic matter is treated with these compounds. More BPDs are being identified all the time. (Richardson et al., 2010) Their concentration may be near the accepted levels for human drinking water but human exposure levels are based on the assumption that a person would drink two liters of water and take a 15 minute bath or shower daily. (Kim, Little, Chiu, & Chiu, 2001; "Trihalomethanes in Drinking-water Background Document for Development of WHO Guidelines for Drinking-water Quality," 2005) Aquatic animals would have a higher exposure due to their more intimate contact with the water at all times. A major route of entry for these compounds is by inhalation so animals that are always in or near water would have more effects from the same amounts considered safe for human use. The athletic nature of animals like sea lions or dolphins would increase respiratory exposure.

Chlorine has been used for water sanitation for about 100 years (Darnall, 1911) and as hypochlorite for about 200 years. (Alcock, 1827) Hypochlorous acid has been a basic biological chemical for much longer. Myeloperoxidase enzymes in neutrophils, eosinophils, phagocytes and lysosomes produce hypochlorous acid or hypobromous acid which can kill bacteria, viruses, parasites and other cells and oxidize tissues and compounds as a basic biological process. Recently myeloperoxidase has been associated with many degenerative and inflammatory diseases in humans including atherosclerosis, kidney disease, asthma, and arthritis. (Pattison & Davies, 2006) (Pullar, Vissers, & Winterbourn, 2000) Chlorination or bromination of biological compounds may play an important role in inflammatory processes. Halogenated organic molecules act as messengers spreading through tissues and stimulating or moderating the effects of inflam-

mation. (Naskalski, Marcinkiewicz, & Drozdz, 2002) When these compounds are used in life support systems they may mimic the natural processes and thereby produce unexpected effects

Oxidants can select for resistant organisms. If all organisms in the water are not killed the ones that survive are the most resistant. Resistant organisms include Mycobacteria, some yeasts, environmental bacteria such as Pseudomonas, some parasites such as Cryptosporidiosis and organisms with resistant spores. Eliminating the normal aquatic bacterial population may give an advantage to these resistant organisms.

A major cause of injury with these compounds is improper use. All of them have significant potential for causing injury if not properly monitored and controlled.

None of these compounds act alone. Combinations of compounds can have very different effects than single compounds. Free radicals produced by UV radiation can add to the effects of some of these compounds. Sunlight on chlorinated water containing bromide will produce bromate in levels higher than allowed for potable water. (Haag & Hoigne, 1983)

Water disinfection by oxidants

Water disinfection has made modern life possible by reducing the incidence of water borne disease. The techniques for production of potable drinking water are well developed. Direct transfer of these techniques to Life Support Systems may lead to unexpected problems. In Drinking water sanitation pre-treatment to reduce total organic carbon (TOC) is required if it is higher than 2 mg/l to reduce formation of disinfection by-products. ("EPA Byproducts Rules," 2010)

Clean water containing 1 mg/l of free chlorine or less appears to be tolerated well by healthy marine mammals. Free chlorine of 0.5 mg/l is more than sufficient to control coliforms in most systems. Recirculating this water between water changes will lead to a build up of waste products from urination, defecation, spilled or uneaten food and environmental additions. Chlorine levels higher than 0.5 mg/l can eliminate biological filtration except in areas with thick biofilm or poorly circulating areas in filters so the wastes will continue to build up to some steady state related to how much water is exchanged daily. I calculated that 4 sea lions living in 60 degree Farenheit water eat as many calories as about 20 humans and don't excuse themselves to go to the rest room! Solid wastes can be removed by particulate filters but the dissolved organic carbon and nitrogenous wastes remain in solution. Until the filters are back-washed the organic debris has not left the system and can continue to add materials to the water as it dissolves or is affected by the oxidants

Chlorine, bromine and iodine the halogens

Chlorine is used in several forms but the most common is hypochlorous acid. It is quite reactive and its potential reactions include substitutions and will replace hydrogen on carbon or nitrogen atoms. It can oxidize the molecule and change it or substitue itself for a hydrogen atom so that the molecule is little changed other than the added chlorine or bromine atom. Halogenation can also occur at Carbon double bonds. Chlorine and bromine atoms are much larger than

hydrogen ions and more electronegative. The addition of the halogen generally makes the molecule more lipid soluble, less volatile and more persistent. the increased lipid solubility may make entry through mucous membranes easier and the persistence may mean higher levels can occur in tissues. Iodinated compounds have recently been studied and found to be cytotoxic at low levels so they may actually be important. (Plewa et al., 2004b)

Recent work has compared the relative toxicity of chlorinated, brominated and iodinated compounds found as BPDs. Nitrogenous BPDs have also been identified and studied. In general the toxicity in the studies done on mammalian tissue cultures and bacterial cultures looking genotoxicity and cytotoxicity showed that Iodinated compounds were more toxic than brominated compounds which were much more toxic than chlorinated compounds. (Plewa, Simmons, Richardson, & Wagner, 2010) Nitrogen containing compounds were more toxic than carbon based BPDs. (Muellner et al., 2007) Nitrogen containing DBPs are more likely to form in water treated with chloramines. Certainly in mammalian life support systems the presence of abundant nitrogenous wastes would increase their formation.

These oxidants are not specific in their reactions. They react with any available substrate. the desired reactions are with bacteria, viruses and parasites to protect against disease. Undesired reactions can produce unexpected BPDs. BPDs include simple compounds such as halogenated methanes but the list of possible compounds is huge. (Richardson et al., 2010) BPDs can have toxic effects. These can be direct or only occur after metabolism produces toxic metabolites. The effects can be chemical but can also have biological mimicking effects or inhibit immune function.

As an example I will discuss the simplest of these compounds which is chloroform or trichloromethane. It is not very toxic until metabolized. It's metabolism starts with initial oxidation by Cytochrome p450 enzyme systems in the cells. For chloroform the initial oxidation produces an unstable product that breaks down to Carbonyl chloride (CCl_2O) and HCl. Carbonyl chloride can react directly with other molecules or even with water to produce CO_2 and 2 HCl. Carbonyl chloride is also known as phosgene and has been associated with industrial accidents and has been used as a poison gas. Damage comes from either the phosgene or the resulting 3 molecules of HCl. When everything in the cell is normal glutathione will bind with the phosgene and reduce it and pH regulating processes will counteract the HCl (hydrochloric acid). The initially minimally toxic chloroform only causes injury to the tissues where the Cytochrome p450 enzymes break it down. This leads to differences in the organs affected in different species and even between male and female related to where Cytochrome p450 enzymes are most active (Bailie, Smith, Newton, & Hook, 1984; Pohl, George, & Satoh, 1984).

The more complex BPDs will also undergo metabolic changes so the ultimate range of possible compounds is huge. Halogenation of amino acids, hormones excreted in urine and feces, and other complex molecules may produce compounds with biological effects other than direct chemical effects.

Another major class of BPDs includes chloramines. In Life Support Systems chloramines can be formed with any free amino group. In discussions of swimming pool chlorination the assumption is that nitrogenous wastes are mainly ammonia. This leads to the use of breakpoint chlorination to attempt to convert ammonia to nitrogen gas which will leave the system.

This does not work in systems with other nitrogenous species and will lead to formation of even more by-products. (Shang, Gong, & Blatchley III, 2000) In inflammation the hypochlorous acid released from the neutrophils allows the formation of chloramines with amino acids and other compounds in the intracellular fluids. Some of these compounds will diffuse from the original site of inflammation and act as inflammatory mediators. Chloramines are still reactive and in fact ammonia chloramines are used as drinking water disinfectants because of their persistence in potable water systems. This allows the reactive capacity of the hypochlorous acid to permeate through the surrounding tissues. Anyone who has had a splinter in their finger can appreciate the inflammation that develops in the the surrounding tissues.

When chloramines are formed with amino acids, and other nitrogenous compounds in life support systems the occurrence of inflammatory reactions like keratitis should not be surprising but should be expected. When the DPD test is done to measure total and combined chlorine and the combined chlorine predominates these compounds exist and their potential for causing injury is related to their concentration and ability to penetrate into tissues. Histidine chloramine is an example. Histidine has buffering capacity and proteins of aquatic animals contain more histidine than similar proteins in terrestrial animals (Abe, 2000). Scombroid poisoning due to the breakdown of histidine to histamine occurs partially because of the high amount of histidine in some species of fish. Excess histidine in the diet will lead to the excretion of histidine in urine. Histidine chloramines do diffuse through intracellular fluid and may be an important inflammatory mediator. (Pattison & Davies, 2005)

Ozone

Ozone is a strong oxidizer. It reacts quickly in water containing organic material. It also reacts with other compounds. In water containing bromide ozone reacts with bromide to form hypobromite BrO-. The hypobromite can react with another ozone to produce either bromide again or to form bromate BrO_3. The cycle of bromide to hypobromite and back catalyzes the destruction of ozone. The bromate does not react much with organic materials but is a suspected carcinogen. Hypobromite forms hypobromous acid and can remain in solution as a residual oxidant or react to from brominated BPDs. (Aiken & Smith, n.d.; Haag & Hoigne, 1983; Keaffaber, n.d.) Sea water normally contains 65 mg/l of bromide and human blood contains 2 to 11 mg/l with older persons and females having higher levels. (Olszowy, Rossiter, Hegarty, Geoghegan, & Haswell-Elkins, 1998) Fresh water contains variable amounts of bromine depending on the terrain the water traverses before use. Some well water and water from gas and oil well operations may have significant bromine levels.

Regulation of ozone dosage is often controlled by ORP readings or ozone sensitive detectors or tests. These are not always correct and significant injuries have occurred to animals where all the readings appeared ok. Careful observation and double checking with DPD tests which are sensitive to residual oxidants reduce the chance of problems. (Aiken & Smith, n.d.; Summerfelt, Clements, & Gearheart, in press) Biological filtration and gas equalization after ozone treatment (Stalter, Magdeburg, Weil, Knacker, & Oehlmann, 2010) is a very valuable safety measure.

Redox levels and Reactive Oxygen Species

To protect tissues against inadvertent effects of these oxidizing compounds a number of protective mechanisms have evolved. Antioxidants such as vitamin C, vitamin E, have protective effects. Glutathione and associated enzymes bind with and remove these reactive compounds. Heme Oxygenase inactivates myeloperoxidase and limits the continued production of these reactive compounds. Membranes surround these compounds in phagosomes and lysosomes to protect the surrounding tissues.

The normal environment inside living cells is a reducing environment. Reactive oxidant species are produced in normal metabolism and are carefully controlled to minimize inadvertent damage to structures and compounds in the cell. Further oxidant stress can over load the mechanisms that maintain the normal redox potential in the cell. In a manner similar to pH regulation in the cell the redox potential must be maintained at a safe level. All oxidizers affect the redox potential in the cell, it is the total sum of oxidizers and reducing agents that gives the redox potential. Oxidizing compounds react by accepting electrons from another molecule so in different circumstances the same compound could act as an oxidant or a reducing agent.

Glutathione is a reducing compound. In cells it is the most important regulator of the Redox level in the cell. Several enzymes help it to react quickly with oxidants and it is oxidized to glutathione disulfide or GSSG. The GSSG is reduced back to glutathione by glutathione reductase using NADPH as an electron donor. The ratio of glutathione to GSSG is the major regulator of the redox level in the cell. In the normal cell oxidative reactions provide the energy for the cell and the glutathione system is active in protecting the cell from the normal energy producing mechanisms. Addition of more oxidants to the system may over load those protective mechanisms. Some BPDs may permanently damage the glutathione molecule so that it cannot be regenerated and there by damage the cell. In the eye this could explain some of the problems seen. "When the intracellular levels of glutathione are reduced in the cornea by one-third, the clarity of the cornea and its ability to pump fluid declines dramatically." from Forrester (Forrester, 2002)

Myeloperoxidase

Myeloperoxidase reacts with hydrogen peroxide to produce hypochlorous, hypobromous and hypothiocyanous acids. The hydrogen peroxide is produced by the respiratory burst which occurs in stimulated neutrophils or phagocytes or by bacteria in some biological situations. The resulting acid will react with other compounds in an oxidation reaction that can be effective in killing bacteria, breaking down biological materials and producing chlorinated or brominated secondary compounds.

Myeloperoxidase has been associated with several degenerative and inflammatory disease states in humans and animal models including arthritis, asthma, renal disease, and atherosclerosis. We may mimic the action of myeloperoxidase when we chlorinate or brominate our systems. In the normal situation the myeloperoxidase and its products are controlled and prevented

from causing damage. External mimics of these processes have no regulation and can continue to stress the antioxidant mechanisms in affected cells

Heme Oxygenase

One of the important regulators of myeloperoxidase is Heme Oxygenase. Heme oxygenase is a stress protein. Its production or activity is triggered by a multitude of stressors including heat, cold, chemical stress, hypoxia, and hyperoxia. It's sole purpose is to break down myeloperoxidase and it produces biliverdin, free ferrous iron, and carbon monoxide (Ryter, Alam, & Choi, 2006) The breakdown of Myleoperoxidase reduces the formation of hypochlorous acid and thereby protects the cell. Interestingly the biliverdin also has an antioxidant effect and the carbon monoxide may have effects on circulation of surrounding tissues. Carbon monoxide added to perfusion fluids for transplanted organs aids in the survival of the organ. In lung transplant studies in mice addition of CO in controlled amounts markedly improves the histologic appearance of tissues compared to those breathing air without any CO. (Ryter et al., 2006)

The implications of this in diving animals where ischemia/reperfusion is a common occurrence may explain the high liver iron levels seen in sea lions and some other animals. Perhaps the oxidants by themselves or via their by-products stimulate this enzyme and cause the release of free iron which is chronically sequestered as part of the protective mechanism in the tissues. Heme oxygenase activity in marine mammals may up regulated in some individuals leading to the high levels of iron sometimes seen.

By-Products of Disinfection (BPDs)

The first BPDs to be reocognized were halomethanes and haloacetic acids. They can be identified by simple gas chromatography and compounds like chloroform and bromoform have been studied for a long time and their potential for causing harm is understood. They are currently regulated in most countries. In The US total trihalomethanes should not exceed 80 ppb. ("EPA Byproducts Rules," 2010) A lot of work has been done on BPDs in recreational pools and each new study using different diagnostic techniques finds more new compounds. (Plewa et al., 2004a; Richardson et al., 2010) While the volatile compounds are regulated and measured studies have shown increased cytotoxicity in tissue culture and bacterial toxicity studies for recreational waters (swimming pools, hot tubs) compared to drinking water even when the volatile BPDs such as trihalomethanes are not different in the recreational waters. (Plewa, Wagner, & Mitch, 2011)

Cytotoxicity studies of BPDs containing iodine, nitrogen, and bromine have recently been performed on recreational and drinking water sources. From these studies cytotoxicity of brominated BPDs are much higher than chlorinated BPDs and iodinated BPDs are more toxic than brominated compounds. Similar studies comparing cytotoxicity of haloacetamides with haloacetonitriles showed the nitrogen containing compounds were significantly more toxic. (Muellner et al., 2007)

There are studies that show that humans drinking chlorinated water have an increase in incidence of bladder and rectal cancers, (McGeehin, Reif, Becher, & Mangione, 1993; Morris, Audet, Angelillo, Chalmers, & Mosteller, 1992) There are many studies to show that swimming

in chlorinated pools increases the risk of asthma. The specific agent causing the asthma is not clear. (Li & Blatchley, 2007) Myeloperoxidase is implicated in asthma as well so the hypochlorous acid may be a common link between the development of asthma in other situations and in asthma resulting from exposure to chlorinated swimming pools. The hypochlorous acid and its BPDs may mimic the effect of myeloperoxidase in several inflammatory conditions. (Pattison & Davies, 2006)

Many of the BPDs resulting from chlorination, ozonation or bromination are unidentified. "More than 50% of the total organic halogen (TOX) formed by chlorination and more than 50% of the assimilable organic carbon (AOC) formed by ozonation has not been identified chemically. The potential interactions among the 600 identified DBPs in the complex mixture of drinking water to which we are exposed by various routes is not reflected in any of the toxicology studies of individual DBPs. " Richardson et al. (Richardson, Plewa, Wagner, Schoeny, & Demarini, 2007) We are dealing with a complex matrix problem when we try to identify what is harmful in such a mass of candidate toxic compounds. The only recourse is to try to reduce them all.

Selection for resistant organisms.

Not all organisms are equally sensitive to oxidants. Mycobacteria, Pseudomonas, yeasts and Cryptosporidium can be found in chlorinated systems. My personal experience with marine mammals in chlorinated water includes a case of Mycobacterium fortuitum, Pseudomonas from ocular lesions, and dermatitis that responded to antifungal treatment after yeasts were seen on histology. Chlorination can kill less resistant organisms but what remains may be a problem. A clean environment with normal ecology is likely to be less supportive of these resistant organisms.

Improper use

Improper use of oxidants is a common cause of problems. Inadequate testing is the easiest mistake to make. If it is not known what the levels are high levels will go unrecognized. Untrained personnel is a common problem. Departure of the usual person in charge means someone unfamiliar with procedures and the the importance of doing things right can easily lead to injury. Broken equipment or failing sensors can easily cause problems with ozone dosing. Plugged injectors on chlorine metering pumps and lead to higher and higher settings until the plug breaks free and high levels result. People without direct animal care and observation duties running the life support system can be a problem. People with direct animal care responsibility should be able to cut back the levels without having to go through administrative channels. Everyone should be watching the animals so if problems develop they can be quickly corrected.

Conclusions

Sanitizing water with oxidants while beneficial in preventing disease may inadvertently produce injury directly by improper use. In water containing organic carbon and nitrogen compounds by-products of disinfection will be produced. The presence of bromide or iodide in the water will produce what appear to be more toxic compounds. The oxidants hypochlorous and hypobromous acids are produced by normal physiologic mechanisms and when we use them we

may mimic or exacerbate normal inflammatory mechanisms. Not all disease organisms are susceptible to these compounds and may be more able to cause disease because competing organisms are reduced.

Recommendations

1. Reduce Total Organic Carbon and Nitrogen compounds in the water

    1. Foam fractionation can remove much of the organic materials in the water and should be considered a prime mechanism for improving water quality. Addition of small amounts of ozone aids flocculation and if followed by biological filtration and gas equalization as in a trickle filter is the safest way to use ozone.

        1. Foam fractionator exhaust should be vented outside. The volatile compounds should not recirculate in a building. If ozone is used this is especially important.

    2. flocculation may help to remove materials more efficiently in particulate filters.

    3. Ultraviolet treatment can significantly reduce dissolved organic carbon and will reduce ozone required for color removal. Exposure to sunlight reduces the cytotoxicity of outdoor pools compared with indoor pools I suspect due to the ultraviolet effect on dissolved organic carbon. (Plewa et al., 2011)

    4. Measuring TOC in sea or salt water is more difficult than in fresh water. The best equipment uses high temperatures and a platinum catalyst to oxidize the the organic carbon compounds to CO2 and then measures the CO2. This equipment is expensive and requires clean gas supplies and careful maintenance.

    5. UV254 is a measurement related to carbon-carbon double bonds and carbon rings in the water. It correlates well with organic carbon compounds that can produce BPDs. It may provide a much less expensive and easier test to use for monitoring. Bromide, nitrite, nitrate and some other compounds can also absorb at 254 nm and must be considered but they do not change as quickly as organic carbon levels.

2. Encourage biological filtration

    1. biological mechanisms represent the best and sometimes only method to remove some compounds. Bromate may be reduced with biological filtration which is almost the only way to remove it.

    2. Nitrogenous wastes are especially important.

    3. Aerated filtration can be very helpful in removing volatile organic compounds

3. Reduce bromine concentrations if possible.

    1. This may be beyond the control of anyone depending on the source water but sea water mixes should not include bromine.

    2. Bromine should not be used as a disinfectant.

4. Aeration

    1. Aeration helps to remove volatile compounds

    2. Helps the efficiency of biological filtration

5. Get particulate matter out of the system quickly to reduce break-up and dissolution

    1. 'No poop should hit a pump' Going through a pump will thoroughly distribute soluble materials and increase the biological material to be removed.

    2. Large skimmer baskets, cyclone separators can be helpful

    3. Design of pools to make solids removal easy is helpful

    4. Sand or bead filters should be back washed more frequently. Waiting until the pressure across the filter rises means all that debris has longer to dissolve and be distributed into the system.

6. Design beaches and haul-outs so urine or feces not actually in the pool will run to waste drains.

7. Reduce the outside organic matter that enters the pools such as leaves, dust, and bird droppings to reduce the addition of organic matter. Don't dump water from empty food buckets into the pools.

8. Consider ultraviolet exposure as an additive stress on sensitive tissues such as the eye and consider shading and colors that do not reflect blue and ultraviolet light.

9. Consider techniques such as pasteurization and membrane filtration to sanitize water without added oxidants. Membrane filtration works well but is expensive. Pasteurization works well in waste water reuse systems for potable water but as far as I know has not been used in Aquarium life support systems. If designed properly using heat from electricity generation or methane from waste sources it could be very cost effective.

10. Consider routine use of nutritional sources of anti-oxidants to maximize the resistance of the animals in the system to the effects of residual oxidants.

11. Consider activated carbon to absorb some of the organic compounds. Activated carbon does not remove everything. If use in chlorinated systems it will remove the chlorine increasing the required chlorine additions. In fish systems there is evidence to suggest that activated carbon increases the risk of Head and Lateral Line Syndrome. (Stamper, Kittell, Patel, & Corwin, 2011) Activated carbon is used as a catalyst in some organic synthesis techniques in

industry so it may have actions other than simple removal of compounds. There are compounds such as bromate that are not absorbed.

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