



We now have over 20 years of field and laboratory experience with the enzyme formula used in **Xbee** Enzyme Fuel Technology. We have conducted many laboratory studies on the effects of this unique product. Our independent laboratory testing has focused primarily on **Xbee's** efficacy in purifying fuel and improving combustion, as well as engine and environmental safety. These tests, as well as field studies conducted by customers and OEM's, give us the empirical proof that **Xbee** is indeed an extraordinary fuel additive.

A great deal of academic research on the mechanistic behavior of enzymes on hydrocarbon substrates was conducted in the late 1970's and 1980's, primarily for oil-spill remediation and for bio-desulfurization of crude oil. In fact, **Xbee** can trace its roots to the industrial sludge treatment and water purification industries. Mechanistic research and the development of the theoretical science about the molecular function of enzymes when suspended in organic solvents like fuels is more recent, and like all novel science, is still advancing...

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## FIELD OF ACTION OF THE XBEE ENZYMES

Combustion testing in all grades of diesel fuel, biodiesel, gasoline and E-10, as well as bunker fuel, demonstrate that **Xbee** improves combustion in all fuels. Several tests have shown that the enzymes in **Xbee** target aromatics. Additionally, **Xbee** appears to be breaking down the large, slow-burning Poly-nuclear Aromatic Hydrocarbons (PAH).

This accounts for the large reductions in soot observed in tests, as well as the reduction in PAH fraction in the exhaust. Aromatics, in particular the PAH molecules, are precursors to soot, and are also the source of carcinogens in diesel exhaust. **Xbee** has also been shown to reduce surface tension in fuel. The surface tension of the fuel has a large impact on the size of the droplets during fuel atomization. As **Xbee** reduces the surface tension, smaller fuel drops are formed, which allows for a faster and more complete vaporization of the fuel charge, and thus a more complete reaction with oxygen. This effect is corroborated by the reduction in CO in combustion testing, regardless of fuel type or aromatic content.

## MECHANISMS OF ENZYME CATALYSIS

There is a significant body of scientific theory on the mechanistic function of enzymes within biological cells, as well as many decades of research on the how extracellular enzymes function in industrial applications such as enhancing petroleum recovery in wells, and soil remediation. The mechanistic function of enzymes in non-polar, organic solvents such as fuel is less studied, but several research papers over the past ten years have described how enzymes suspended in organic solvents can catalyze novel reactions that are impossible in aqueous solutions.

Enzymes are catalysts which lower the activation energy of a reaction by providing a pathway(s) with lower activation energy than in a non-enzymatic reaction. For many years, a simple "lock and key" theory was used to describe enzyme-substrate specificity and function. This simple model can still be used, but with a major stipulation: the substrate is not the "key". The key is the "Transition State". Generally, enzymes collide with substrates as they diffuse through the fuel, although some may be further attracted to substrates (target molecules) by electrostatic charges.

They contact their target molecules, and form Enzyme-Substrate complexes, called the "ES". Enzymes alter the electrical charges on substrates, temporarily binding to them. Between the starting point; a substrate binding to an ES, and the release of the product, there is a temporary state called a transition state, which can be described as a high-energy structure somewhere between the reactant and product. After the ES is formed, the enzyme distorts the substrate, forcing it to the transition state. It is the transition state, rather than substrate, that is the key.

Protein folding at receptor site with hydrocarbon bond



Three-dimensional structure of isomerase enzyme



The substrate reacts only transiently with the enzyme. After catalysis, or "conversion", the product is released and the enzyme is free to perform more reactions. Some enzymes, under the right conditions, can perform catalysis as fast as the enzyme can diffuse into the solution. Reaction rate acceleration can be as high as  $10^{17}$  faster than non-enzymatic reactions. One recent researcher has identified some enzymes could be as much as  $10^{21}$  faster. Enzymes can perform cleavage reactions, as previously described with PAH molecules. Hydrocarbons with covalent bonds (where pairs of electrons are shared by two atoms) are separated by the enzyme, with either one atom retaining both electrons, or both atoms each retaining just one electron. The substrate, once a large, difficult to burn molecule, is converted to two smaller molecules. To the engine, this means the fuel is more easily evaporated during injection, and can be ignited easier and react with more oxygen to burn more completely.

The enzyme's amazing speed at catalysis stems from binding modes. The binding forces behind ES complexes are charge-charge interactions, hydrogen bonds, hydrophobic interactions, and van der Waals forces. Charge-charge interactions are stronger in non-polar environments such as organic solvents than in water. It is believed that **Xbee's** extraordinary speed, efficacy, and longevity are derived from this phenomenon. The substrate affinity and speed of catalysis are dependent upon the three-dimensional structure of the enzyme. Enzymes evolved to function in aqueous solutions and the position of individual water molecules within the enzyme's active sites are critical to both substrate sensitivity as well as the rate of catalysis. A monolayer, perhaps less than 500 water molecules, are always present, permanently attached to the surface of the enzyme. It is believed that when suspended in a non-polar, organic solvent, the hydrophobic force of the solvent pushes on the water molecules, in effect, forming a cage that holds the enzyme in its three-dimensional shape. This allows the enzyme to function over extraordinarily long periods of time, as well as improve its thermal stability.

Several thousand enzymes have been identified in animals. While most textbooks discuss the highly specialized actions of enzymes, known as "substrate specificity", this is a derivative of enzyme research having been financed primarily for human or animal biological studies. Plant enzymology is less studied. Plants evolved roughly five hundred million years before animals, at a time when the prehistoric soil was relatively barren of organic matter. Plants typically produce only a few dozen different enzymes, some of which evolved to catalyze multiple substrates in order to secure nutrients critical to their growth. This phenomenon is known as "enzyme promiscuity". Over two dozen plant enzymes are used in **Xbee** which provides a wide range substrate reactivity.

As previously noted, **Xbee** has surfactant properties. Standardized ASTM testing has demonstrated this phenomenon in petroleum fuels. Recently, other researchers working with enzymes within the same class as those used in **Xbee** have demonstrated that enzymes can work in concert with each other, resulting in a surfactant affect that is collectively stronger than the individual enzymes. These enzymes break down sludge and disperse microscopic drops of water during fuel agitation. An enzymatic bio-surfactant differs from the chemical surfactants used in detergents in that they never become permanently bonded to the substrate. Enzyme breaks the electrical bonds holding the sludge to the fuel tank walls, and helps dissolve the sludge into smaller complexes, as would a detergent. The enzyme then releases from the dissolved sludge, or "products", and is free to act over and over, almost indefinitely. Chemical surfactants act only once, and become bonded to the product. Therefore they have a finite reach and lifespan in fuel. Far fewer enzyme molecules are required to do the same amount of cleaning work as many times their amount of chemical surfactants. This eliminates the potential precipitation of chemicals that occasionally plagues conventional fuel additives if overdosed.

### EFFECT ON EMISSIONS

As mentioned previously, **Xbee's** cleaving action reduces the size of the larger aromatic hydrocarbons that are precursors to soot. Aromatics have an opposite relationship to cetane index and number, and fuels that have high aromatics are typically lower in cetane ratings compared to fuels with low aromatics. **Xbee** can significantly increase cetane ratings in fuels by reducing these larger aromatics. This function helps improve cold-starts, as well as generally improves combustion, and reduces the most toxic exhaust fractions; the PAH molecules.

Additionally, **Xbee's** effect on reducing surface tension results in smaller fuel droplets and faster evaporation. When combined, these two effects result in hyper-oxygenation of the fuel as it is injected into the combustion chamber. This leads to a better and more complete combustion. Emissions tests demonstrate this effect through a reduction in CO and HC, including the toxic PAH fractions. Less soot results in a reduced rate of soot contamination in the lube oil, as well as preventing hardened carbon deposits from forming in the combustion chambers.



As concussive forces in the engine slough off old carbon deposits, combined with **Xbee's** action of preventing carbon deposits from forming, engines are cleaned over a period of time. An endoscopic study of a commercial marine engine by OEM Wärtsilä confirms these benefits result from **Xbee's** improved combustion.

Inside the combustion chamber, the nitrogen in the air is oxidized into NOx. Higher combustion chamber temperatures result in higher NOx. By improving the rate of vaporization and ignition characteristics, **Xbee** gives the fuel charge more time to fully burn. This reduces or eliminates the amount of still-burning fuel that contacts the cylinder walls or is pushed past the exhaust valves, super-heating them. This tends to reduce NOx, even as the CO, HC, and soot particles are reduced.

During combustion testing, **Xbee** has exhibited an additional effect; the ability to reduce SOx. Currently, there is no conclusive science explaining **Xbee's** observed reduction in SOx emissions other than the obvious reduction from improved combustion and reduced fuel consumption. Over a time-weighted study such as an emissions test, there is less SOx because less sulfur-bearing fuel went into the engine over the course of the test.

In some tests, SOx reductions are beyond the reduction in fuel consumption. This is not understood. There are two prevailing theories. In fuels with high levels of sulfur-bearing molecules, the enzyme cleaves some of these molecules, leaving a portion of the sulfur free. Atmospheric oxygen present in fuel tanks is believed to react with the elemental sulfur, forming sulfur oxides that are then reacting with water in the fuel. The new molecules are denser, and gravity separate. The water, containing trace amounts of sulfur, is collected in water traps and is thus prevented from being combusted.

Another theory is that after the enzymes have acted upon the sulfur-bearing molecules, after the combustion cycle of the engine, the small un-combusted portion tends to form sulfites or sulphate particles, rather than react with oxygen to form SOx. These particles would be discharged as a small amount of solid particles in the exhaust, and are not detected by standard SOx testing.

## EFFECT ON BIOMASS

Bacteria and fungi are present throughout the environment. Many species can utilize the carbon in fuel for energy. All fuels, but particularly low-sulfur fuels and biofuels, are an excellent food source for microbial growth. During refining, a process called hydro-treating breaks down the sulfur-bearing hydrocarbon molecules. This allows the refiner to remove the sulfur as it lowers soot-forming aromatics, and together, this process allows the refiner to obtain a higher yield of cleaner burning fuel from each barrel of crude oil. Unfortunately, the elimination of sulfur has two negative results. First, the sulfur-bearing hydrocarbons provided diesel fuel with natural lubricity. To restore lubricity, the refinery may add a chemical lubricity agent. These additives are polar, meaning they will attract moisture. For economic and environmental reasons, Fatty Acid Methyl Ester (FAME, also known as biodiesel) is mandated in much of the diesel fuel around the world and as FAME is an excellent lubricity agent, this is often the lubricity agent of choice. FAME is also polar and, it too, attracts moisture into the fuel. Water is necessary to support infestations.

Second, sulfur is a natural biocide. Its removal makes the fuel more prone to microbial infestation. With no natural biocide, and fuel now being hygroscopic, the incidence of microbial infestations and microbial influenced corrosion (MIC) in diesel has skyrocketed. An additional development in parts of the world has recently been uncovered. Ethanol, which is used as an additive in E-10 gasoline (petrol) or as an alternative fuel (E-22 and E-85) is often cross-contaminating diesel fuel during switch-loading. This is when diesel fuel is delivered in the same tankers as ethanol fuels, and the hoses are not thoroughly cleansed before fuel is switched. Further, highly volatile ethanol passes from its compartment through the venting system of the tanker to the non-volatile diesel, where it is absorbed. In 2016, in an extensive US EPA study, ethanol was found in 90% of the fuel tanks. Ethanol is broken down by bacteria of the acetobacter species and converted to acetic acid. This is the acid in vinegar. The volatile acid vapors rise to the fuel tank's ullage (vapor space) and rapidly corrode the metal components. The EPA study found 83% of the fuel tanks had moderate to severe rust, with the most severe damage being in the vapor space.

Although distillate fuels are sterile at their completion, with every transfer of fuel from a bulk tank to a pipeline or tanker, then the transfer to a service station or fuel wholesaler, then another transfer to the customer's fuel tank, the fuel must be vented to the air to prevent a vacuum. As air enters the fuel system, so does moisture and microbes, as well as dust and dirt. Even fuel in back-up tanks is exposed, as during the day, the fuel heats up and expands, driving out air. At night as the fuel cools, it contracts, sucking in air, along with contaminants. This was not a serious problem until the implementation of ultra-low sulfur diesel, and biofuels like FAME and ethanol.



Biocides have been used for 50 years to control fungal growth. They are toxic to both the environment and the mechanic that gets diesel fuel on his hands in the shop. These can be harsh pesticides. No engine is perfect in burning all its fuel, and pesticide is spewed into the air we breath, and the water we drink. The better solution is to use **Xbee** enzymes for breaking water emulsions, dismantling fungal growth, and the dissolving of petroleum based sludge.

In effect, the enzyme is really nothing more than an organically obtained surfactant (detergent) which neutralizes charged atoms and keeps them from clinging to each other or to other surfaces. One benefit of using Xbee will be to disperse a small quantity of water on a microscopic level during fuel movement and eliminating it during combustion. As the water is removed from the fuel, microbial spores will not thrive, thus preventing filter plugging and MIC.

As corrosion in the vapor space of diesel tanks was not common until the implementation of ULSD and ethanol, there is no ASTM standardized test for additives to prevent vapor space corrosion. A 2013 test designed in-house by a petroleum engineer for a marine consumer report (Practical Sailor, August 2013) found that out of the largest national marine fuel additive brands, our enzyme technology was the best at preventing corrosion in contaminated diesel, and the only additive that protected in the vapor space.

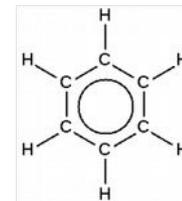
Along with Ultra low sulfur diesel and biofuels, an additional change in diesel engines, the implementation of High Pressure Common Rail fuel systems, has changed fuel housekeeping necessities. With fuel pump pressures now commonly over 30,000 PSI, most OEM's have added fuel filters with pores as small as 4 to 5 microns. Some have adopted filters as low as 2 microns, and are considering the possibility of 1 micron in the future. Today, fuel must be nearly free of particulates. Warranties insist on fuel meeting ISO 4406, a rating standard for 4, 6, and 14-micron particulates. The intent of this specification, and the smaller filters, is to protect small fuel injector orifices from hard particles like rust, silicate, and dirt. However, soft organic particulates like gums and biomass can coat the filters, preventing them from passing clean fuel to the engine.

Testing has demonstrated that the enzymes in **Xbee** dissolve organic contamination, and can significantly reduce the organic particulates as measured under ISO 4406, even in old, oxidized fuel. This enzymatic action is the same action that provides **Xbee** with its stabilization function. Not being self-limiting like a chemical additive, **Xbee**'s effects on preventing or reducing gums and particulate can last for months, or even years in fuel stored at ambient temperatures.

## LEXICON

- *Aromatic compounds*

An aromatic hydrocarbon, also known as aromatic compound or aromaticity, contains a set of covalently-bound atoms with specific characteristics, such as benzene (six electrons, from three double bonds). This is usually considered to be because electrons are free to cycle around circular arrangements of atoms, which are alternately single- and double-bonded to one another. These bonds may be seen as a hybrid of a single bond and a double bond, each bond in the ring identical to every other.



Benzene [C<sub>6</sub>H<sub>6</sub>]

- *Catalysis (enzyme)*

It is the catalysis of chemical reactions by specialized proteins known as enzymes. Catalysis of biochemical reactions in the cell is vital due to the very low reaction rates of the uncatalysed reactions. The mechanism of enzyme catalysis is similar in principle to other types of chemical catalysis. By providing an alternative reaction route and by stabilizing intermediates the enzyme reduces the energy required to reach the highest energy transition state of the reaction. The reduction of activation energy increases the number of reactant molecules with enough energy to reach the activation energy and form the product.

- *Coenzymes*

They are small organic non-protein molecules that carry chemical groups between enzymes. Coenzymes are sometimes referred to as cosubstrates. These molecules are substrates for enzymes and do not form a permanent part of the enzymes' structures. This distinguishes coenzymes from prosthetic groups, which are non-protein components that are bound tightly to enzymes. Both coenzymes and prosthetic groups are types of the broader group of cofactors.

- *Cofactors*

A cofactor is a non-protein chemical compound that is bound (either tightly or loosely) to an enzyme and is required for catalysis. They can be considered "helper molecules/ions" that assist in biochemical transformations. Certain substances such as water and various abundant ions may be bound tightly by enzymes, but are not considered to be cofactors since they are ubiquitous and rarely limiting. Some sources limit the use of the term "cofactor" to inorganic substances.

- *Convection*

Convection is one of the major modes of heat transfer and mass transfer within fluids to the surface.

- *Covalent bond*

This is a form of chemical bonding that is characterized by the sharing of pairs of electrons between atoms, or between atoms and other covalent bonds. In short, attraction-to-repulsion stability that forms between atoms when they share electrons is known as covalent bonding.

The most common type of covalent bond is the single bond, the sharing of only one pair of electrons between two atoms. It usually consists of one sigma bond. All bonds with more than one shared pair are called multiple bonds: sharing two or three pairs is called a double bond or a triple bond.

The triple bond is quite rare in the nature, in Carbon Monoxide for instance. A special resonance case is exhibited in aromatic rings of atoms (for example, benzene). Aromatic rings are composed of atoms arranged in a circle (held together by covalent bonds) that may alternate between single and double bonds according to their Lewis Dot Structure. In actuality, the electrons tend to be disambiguously and evenly spaced within the ring. Electron sharing in aromatic structures is often represented with a ring inside the circle of atoms.

- *Enzymes*

Biomolecules that catalyze chemical reactions. Almost all enzymes are proteins synthesized by living organisms (tree leaves and marine algae in that case). Almost all processes in a biological cell need enzymes to occur at significant rates. Since enzymes are selective for their substrates and speed up only a few reactions from among many possibilities, the set of enzymes made in a cell determines which metabolic pathways occur in that cell.

- *Exothermic reaction*

A reaction is considered as exothermic when it releases energy in the form of heat. The rate of reaction can increase, in turn causing heat to be evolved even more quickly. An explosion can result from the problem.

- **Ligand**

In biochemistry, a protein ligand is an atom, a molecule or an ion which can bind to a specific site (the binding site) on a protein. Interactions between any protein and its ligands are fundamental and essential for the protein to function properly.

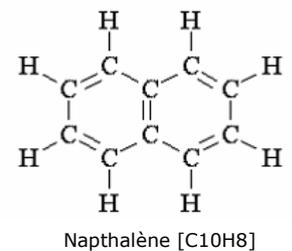
- **Mass spectrometry (MS)**

It is an analytical technique for the determination of the elemental composition of a sample or molecule. The MS principle consists of ionizing chemical compounds to generate charged molecules or molecule fragments and measurement of their mass-to-charge ratios.

In a typical MS procedure, a sample is loaded onto the MS instrument, and its compounds are ionized by different methods (e.g., by impacting them with an electron beam), resulting in the formation of charged particles (ions). The mass-to-charge ratio of the particles is then calculated from the motion of the ions as they transit through electromagnetic fields.

- **Polycyclic or poly-nuclear aromatic hydrocarbon (PAH)**

Chemical compounds that consist of fused aromatic rings and do not contain heteroatoms carry substituents. PAHs occur in oil, coal, and tar deposits, and are produced as byproduct of fuel burning (whether fossil fuel or biomass). As a pollutant, they are of concern because some compounds have been identified as carcinogenic, mutagenic, and teratogenic. PAHs are also found in cooked foods, such as grilled meats, or in tobaccos.



- **Product**

Molecule that forms as a result of an enzyme catalysis reaction.

- **Substrate**

Molecule upon which an enzyme acts. The substrate binds with the enzyme active site, and an enzyme-substrate complex is formed. The substrate is broken down into a product and is released from the active site. The active site is now free to accept another substrate molecule.

- **Surfactant**

Surfactants reduce the surface tension of water by absorbing at the liquid-gas interface. They also reduce the interfacial tension between oil and water by absorbing at the liquid-liquid interface. Many surfactants can also assemble in the bulk solution into aggregates.

The term surfactant is a blend of surface acting agent. Surfactants are usually organic compounds that are amphiphilic, meaning they contain both hydrophobic groups (their "tails") and hydrophilic groups (their "heads"). Therefore, they are soluble in both organic solvents and water.

- **Surface tension**

It is an attractive property of the surface of a liquid. It is what causes the surface portion of liquid to be attracted to another surface, such as that of another portion of liquid (as in connecting bits of water or as in a drop of mercury that forms a cohesive ball).